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PLANT PHYSIOLOGY

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THE ESTIMATION OF DEHYDROGENASES IN PLANT TISSUE[1, 2]

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Although a great deal of information has been amassed concerning dehydrogenases in animal tissues, there was for a long time little evidence that certain of these important enzymes even existed in plants. Malic and citric dehydrogenases were reported in 1929 in cucumber seeds (41), but it was not until 1939 that succinic dehydrogenase was found, first in pollen by Okunuki (28, 29) and then in certain other tissues (9, 11, 14). Nevertheless, the apparent absence or near-absence of succinic dehydrogenase in some tissues (2, 3, 6, 14, 42) as well as the occasional reports of the presence of individual enzymes (10, 23, 41, 42) seemed to indicate that the dehydrogenases, at least those of the 4 carbon and 6 carbon acids, were distributed only sporadically. It was during this period that the tricarboxylic acid cycle of Krebs (19), embodying many of these dehydrogenases, was becoming accepted as the main pathway of respiration in animal tissues. Respiration studies in plants (1, 5) pointed in the same direction.

In the last two years, the situation has greatly altered: the survey of Bhagvat and Hill (4), and the reports of Price and Thimann (32) on oat and pea seedlings and of Millerd (24) on potato tuber have shown that the earlier negative findings on succinic dehydrogenase were not justified, and have implied its general distribution in plants. Conn et al (8) have brought to light the enzyme causing simultaneous dehydrogenation and decarboxylation of malic acid and shown its wide distribution in plants; and most recently (20, 25, 26, 38), great progress has been made in identifying plant systems corresponding to the animal cyclophorase.

While the occurrence of the enzymes is thus no longer in doubt, their properties and their concentrations in plants remain little known. As to their properties, there is evidence that the methods of animal enzymology cannot always be employed unchanged in the study of plant dehydrogenases. For example, Laties (21) has discussed the special osmotic requirements of enzymatic particles from cauliflower, and our own studies (32) showed that the succinic dehydrogenase of oat coleoptiles is not only highly labile but is inactive toward methylene blue and thionin, dyes widely used with the animal enzyme.

[1] Received May 29, 1953.

[2] Supported in part by a research grant from the Committee on Growth of the American Cancer Society.

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METHODS AND MATERIALS

PREPARATION OF ENZYMES: Since enzymes appear to alter in amount and type within a given tissue according to age, conditions of growth, and other factors, plant material capable of rigid standardization was selected: the apical 30 to 50 mm of 7-day-old etiolated pea stems (*Pisum sativum* var. Alaska) and the coleoptiles of 72-hour etiolated oat seedlings (*Avena sativa* var. Segerhavre). The conditions for growth were essentially those for studies of auxin-induced growth (39). The pea stem preparation served for many of the dehydrogenase experiments and for all of the oxidase manometric work. Except for the greater sensitivity to inactivation shown by the *Avena* particles, no qualitative differences were found between the enzymes from the 2 tissues.

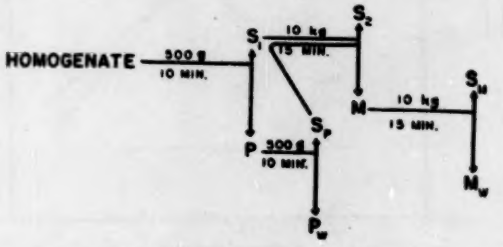


FIG. 1. Scheme of centrifugal fractionation. The inclusion of S_p in the first high-speed centrifugation is essential for quantitative recovery of M. The fraction used in subsequent experiments is M_w.

Extraction and purification were conducted in a 3°C coldroom. Chilled tissue was ground in 1.5 to 10 times its weight of a solution 0.2 M in sucrose and 0.03 M in phosphate buffer of pH 7. A Potter-Elvehjem tissue grinder was employed for tissue weighing up to a gram, and a mortar and pestle for larger quantities. The macerate was then washed through a coarse sintered glass funnel to remove large cell debris.

Fractional centrifugation was accomplished by a modification of the widely accepted method of Hogeboom, Schneider, and Palade (17) as summarized in figure 1. A low-speed (500 to 1000 × g) sediment containing plastids, starch, and probably nuclei, was designated P ("plastid"); the washed plastid fraction

was termed P_w. For quantitative recovery, it is essential to return the supernatant, S_p, from the plastid wash for further centrifugation (fig 1). The second, or mitochondrial,[4] fraction, M, was almost completely precipitated below 8000 × g. From 8000 to 20,000 × g very little was sedimented. Material remaining in the supernatant after centrifugation at 10,000 × g (S_2 in figure 1) was not examined. Each sediment was washed with sucrose-phosphate mixture and resuspended by means of a small mechanical homogenizer. It was found beneficial to protect the mitochondria after the first wash by adding bovine serum albumin, 3 mg per ml solution.

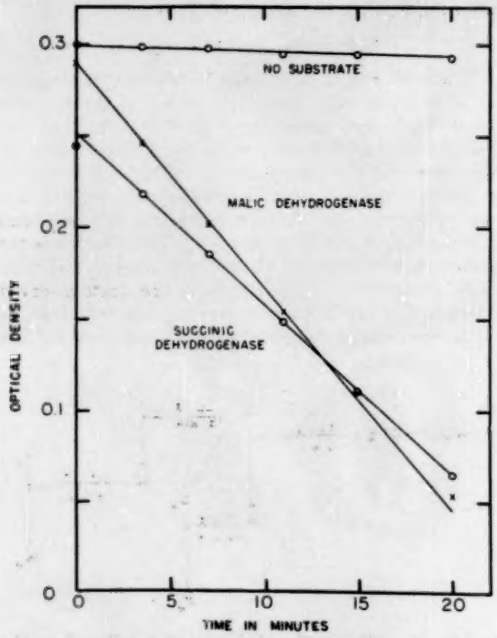


FIG. 2. Time course of dehydrogenation in the Thunberg tube. Malic and succinic dehydrogenases measured with particles (M_w) from 10 and 100 mg, respectively, fresh weight of oat coleoptiles.

Finally the sediments were diluted with sucrose-phosphate mixture to contain per ml the equivalent of either 100 mg fresh weight (FW) of tissue for the Thunberg dehydrogenase analyses or 1 to 3 gm fresh weight of tissue for the manometric oxidase determinations.

DEHYDROGENASE DETERMINATION: Although now used somewhat less than formerly, the Thunberg method has two advantages: it limits the number of enzymatic reactions under study, and it can yield quantitative results with very small amounts of plant tissue.

[4] This solution was selected to give optimal oxidase activity, and not less than optimal dehydrogenase activity (see under Thunberg and manometric methods below).

The enzyme was added to Thunberg tubes chilled in cracked ice and evacuated by a mechanical pump with vigorous shaking. The tubes were then equilibrated 5 minutes in a 25°C bath before the contents were mixed.

The tubes were constructed with shanks which fitted into a Klett-Summerson Colorimeter; readings of optical density were taken at 2- to 5-minute intervals for 20 minutes. Figure 2 is a plot of such readings against time. This method allows the use of such dilute suspensions that settling of the particles does not occur, yet light scattering is negligible. This direct determination of the linear rate of bleaching is very greatly preferable to the usual estimation by dye decolorization time.

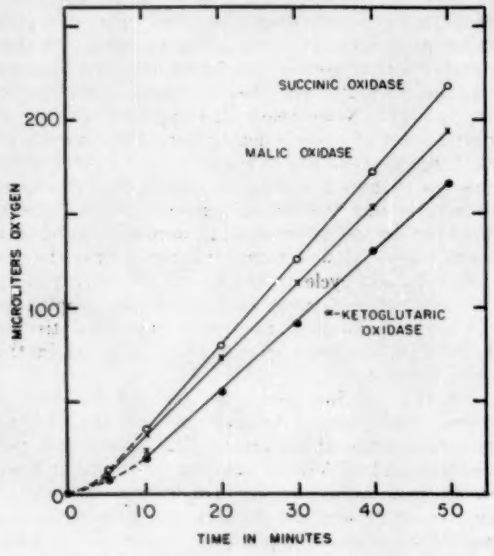


FIG. 3. Time course of oxidation in Warburg vessels. Oxidase activity measured with particles (M_w) from 3 grams fresh weight of pea stem internodes.

While this method determines the rate of hydrogen transfer, directly or via a flavoprotein, to the ultimate acceptor, to convert the rate to terms of oxygen uptake it is necessary only to know the molecular weight of the dye and its redox equivalent. In the case of 2,6-dichlorophenolindophenol, the molecular weight (of the sodium salt) is 290 and the equivalent is 2. Empirically it was found that 1.0 unit of optical density corresponds to the reduction of 0.53 μM of dye and is, therefore, equivalent to the utilization of 13 μl of oxygen.

The activities of the enzyme preparations described herein were of the order of $Q_{O_2}{}^N = 300$ for succinic dehydrogenase and = 1000 or higher for malic dehydrogenase.

The concentrations of components for succinic and malic dehydrogenase preparations are listed in the following protocol:

Component	Final Concentration
Succinate	0.025 M
Phosphate, pH 6.8	0.05 M
Dichlorophenolindophenol, 0.5 ml	0.0001 M
Bovine serum albumin	0.2 mg per ml
Washed particles (M_w)	50 mg FW per tube
Water	to 4.0 ml

Malate	0.025 M
Phosphate, pH 7.8	0.05 M
Coenzyme I	0.001 M
Sodium cyanide	0.01 M
Dichlorophenolindophenol, 0.5 ml	0.0001 M
Bovine serum albumin	0.2 mg per ml
Washed particles (M_w)	10 mg FW per tube
Water	to 4.0 ml

OXIDASE DETERMINATION: Oxygen uptake was measured in the Warburg vessel by conventional methods. The enzyme preparation was added to chilled vessels and equilibrated for 5 to 10 minutes in a 25°C constant temperature bath. Readings were plotted against time (fig 3) for an hour or more, and linear rates of O_2 uptake for 30 minutes were taken as a measure of enzyme activity; these rates were then converted to microliters O_2 uptake per hour. The rates averaged 300 to 600 μl O_2/hr × mg N.

The final concentrations of components found optimal for the 3 oxidases are listed in the following protocol:

Component	Final Concentration
α-Ketoglutarate	0.02 M
Phosphate, pH 7.0	0.04 M
Magnesium sulfate	0.001 M
ATP	0.001 M
Sucrose	0.2 M
Bovine serum albumin	3 mg per ml
Washed particles (M_w)	1 to 3 gm FW per vessel
Water	to 1.5 to 3.0 ml

Succinate	0.02 M
Phosphate, pH 7.0	0.01 M
Sucrose	0.2 M
Bovine serum albumin	3 mg per ml
Washed particles (M_w)	1 to 3 gm FW per vessel
Water	to 1.5 to 3.0 ml

Malate	0.02 M
Phosphate, pH 7.15	0.005 M
Coenzyme I	0.001 M
ATP	0.001 M
Magnesium sulfate	0.001 M
Sucrose	0.2 M
Bovine serum albumin	3 mg per ml
Washed particles (M_w)	1 to 3 gm FW per vessel
Water	to 1.5 to 3.0 ml

MATERIALS: Glass-distilled water was used throughout. Alpha-ketoglutaric acid, m.p. 115 to 116°C, with the correct neutralization equivalent and free from succinate, was prepared by a modification of earlier methods (31). Phosphate buffers were made up from KH_2PO_4 and Na_2HPO_4. Bovine serum albumin was purchased from Armour and Company. Cytochrome c, 65 % coenzyme I (corrected to pure Co I throughout), adenosine triphosphate, and Sequestrene (a thrice-recrystallized preparation of the disodium salt of ethylenediaminetetraacetic acid) were commercial samples.

Alpha-keto acids were determined by the method of Friedemann and Haugen (12). Succinate was determined with the ox heart succinoxidase assay according to Umbreit et al (43).

The following abbreviations will be used: ATP (adenosine triphosphate), BSA (bovine serum albumin), DIP (2,6-dichlorophenolindophenol), MB (methylene blue), and FW (fresh weight of original tissue).

EXPERIMENTAL RESULTS

THUNBERG METHOD: Succinic and malic dehydrogenases could be readily demonstrated by the method outlined, but no dehydrogenase could be detected for pure α-ketoglutaric acid. Furthermore, the dye was not reduced by plant particles under the conditions described for the α-ketoglutaric dehydrogenase of pig heart (33). Commercial preparations of α-ketoglutaric acid contain appreciable amounts of succinate (31) which rapidly reduce the dye, and in addition some samples of the acid contain an orange impurity which decolorizes DIP non-enzymatically.

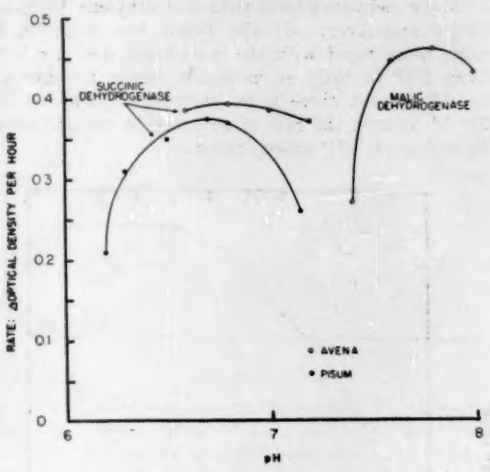


FIG. 4. Effect of pH on succinic and malic dehydrogenase activity.

Glass-distilled water was found to be the best extracting medium. The addition of sucrose beyond 0.4 M, or phosphate beyond 0.05 M, led to decreased activity; inert protein (100 mg/l BSA) produced no effect. In 0.01 M phosphate, of pH 5.5 to 7.5, identical succinic dehydrogenase activities were recovered; at pH 5 and pH 8 the recovery was depressed.

When particles obtained by fractional centrifugation (M_w of figure 1) were maintained at 3° in the following solutions for 60 minutes, no loss could be detected: phosphate up to 0.05 M, sucrose up to 0.6 M, and BSA at 100 mg/l. This shows that the

60 minutes required for preparation entails no loss in activity (36).

The pH optimum of succinic dehydrogenase was found to be 6.8 (fig 4) which agrees with that of the enzyme from potato tuber (24). The pH optimum was found to be essentially independent of the dye concentration.

The pH optimum for malic dehydrogenase is 7.8 (fig 4) which falls on a plateau previously reported for this enzyme by Berger and Avery (3), although the pH-dependence appears to be sharper than was reported by these workers.

The absence of succinic dehydrogenase in the *Avena* coleoptile (3) was probably due to the choice of redox dye, as mentioned earlier (32). The oat and pea enzymes not only fail to decolorize methylene blue and thionin, but are strongly inhibited by one of them (MB) as shown in table I.

Malic dehydrogenase reduces methylene blue, as Berger and Avery (3) also found, but oxidation is much more rapid with the indophenol dye (fig 5a). Even DIP is toxic at moderate concentrations; at 10^{-3} M dilution there is no enzyme activity, but at 10^{-4} M dilution the rate of reduction is not critically dependent on DIP concentration.

TABLE I

EFFECT OF METHYLENE BLUE (MB) AND DICHLOROPHENOLINDOPHENOL (DIP) ON DEHYDROGENASE ACTIVITY. ENZYME ACTIVITY (V) EXPRESSED AS DECREASE IN OPTICAL DENSITY PER HOUR

SUBSTRATE	MB	DIP	V	% DIP CONTROL
	Molar	*Molar*		
Succinate	0	14^{-4}	0.200	..
"	2×10^{-5}	10^{-4}	0.192	96
"	1×10^{-5}	10^{-4}	0.180	90
"	5×10^{-5}	10^{-4}	0.122	61
"	1×10^{-4}	0	0	0
Malate	1×10^{-4}	0	0.192	..
"	0	10^{-4}	0.734	..
α-Ketoglutarate	1×10^{-4}	0	0	..
"	0	10^{-4}	0	..

The Lineweaver and Burk (22) double reciprocal plot of dehydrogenase activity against substrate concentration departs slightly from the linear with succinic dehydrogenase (fig 6). Depending on the substrate concentration, the calculated K_s varies from

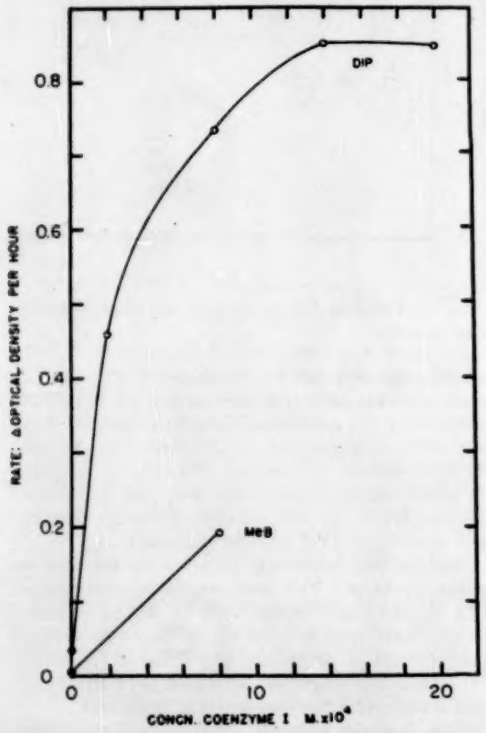


FIG. 5a. Saturation of malic dehydrogenase with coenzyme I. With DIP as redox dye, coenzyme I is non-limiting at 1.4×10^{-4} M. With MB as dye the reaction is strongly inhibited.

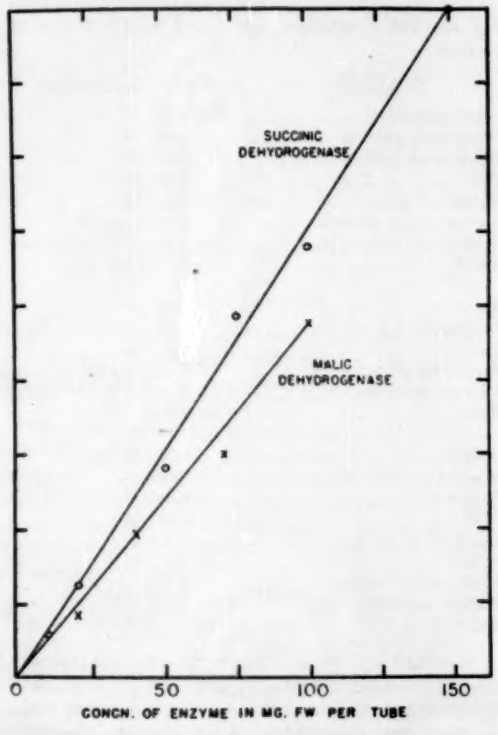


FIG. 5b. Proportionality between enzyme velocity and enzyme concentration. In practice the Thunberg method is found to be quantitative over a 20-fold range of activity.

0.00023 M to 0.00059 M, subject to the reservations pointed out by Slater and Bonner (37). These K_s values indicate a greater enzyme-succinate affinity than any previously reported, save that for *Neurospora* (36). The enzyme from potato tuber (24) has a K_s of 0.019 M.

Malic dehydrogenase, as already shown (3), manifests an almost absolute requirement for coenzyme I (fig 5a); the enzyme is saturated at somewhat more than 10^{-3} M. Such data as can be found (35) suggest that this value is a little higher than for other coenzyme-requiring dehydrogenases.

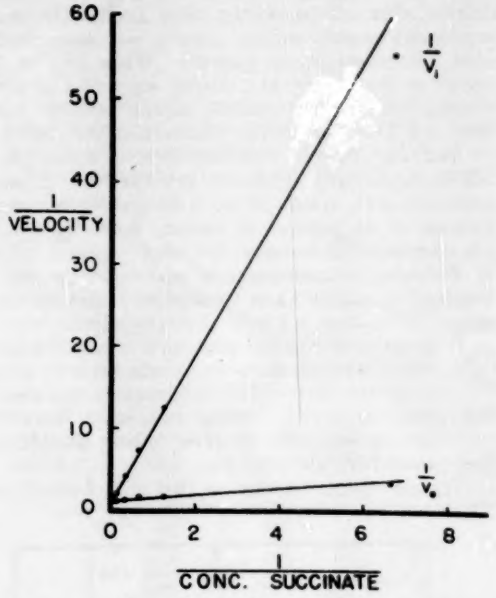


FIG. 6. Competitive inhibition of succinic dehydrogenase by malonate. Lower curve, succinate alone; upper curve, in presence of 5×10^{-4} M malonate. From this and similar Lineweaver-Burk plots of enzyme velocity as a function of substrate concentration, the K_s for succinic dehydrogenase of oat coleoptiles is found to range from 0.00023 M to 0.00059 M. The K_i for malonate is 0.0019 M.

With respect to phosphate concentration in the reaction mixture, the rates of succinic and malic dehydrogenases are unaffected from 0.01 to 0.1 M. At lower concentrations malic dehydrogenase activity falls rapidly, an effect which may be due to impaired buffering, since the substrate offers no effective buffering at pH 7.8. Succinic dehydrogenase remains constant at phosphate concentrations as low as 0.001 M, as has been reported by several workers for the corresponding enzyme from animal tissues (7). It must be borne in mind that mitochondria contain phosphate (15).

Similarly, no benefit is derived from the addition of sucrose to the reaction medium. In fact, malic dehydrogenase appears to be inhibited by sucrose above 0.4 M, but this might be due to the increased viscosity affecting the efficiency of evacuation.

The lack of any real effect of these 2 non-specific agents, namely phosphate and increased osmotic pressure, on the dehydrogenase activity of plant particles constitutes an important difference between the requirements of enzymatic dehydrogenation when measured directly and when coupled through the cytochrome system to O_2 (see below).

As noted above, it was found unnecessary to add protective agents to maintain full activity during short-term storage at 3°C. During the reaction in the Thunberg tube, however, the particles are diluted and raised to 25°C. This may be the reason for an observation, confirmed many times, that the dehydrogenase activity of washed particles is low unless stabilized by one of a number of non-specific agents incorporated into the reaction mixture: gelatin, ovalbumin, BSA,

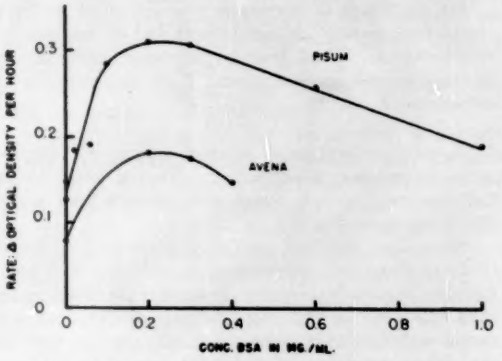


FIG. 7. Effect of protein on succinic dehydrogenase activity. Bovine serum albumin can be replaced by a number of proteins and, to some extent, by Sequestrene.

a globulin prepared from germinating pea endosperm, asparagine, or the chelating agent, Sequestrene. The latter duplicates the effect of BSA on pea stem particles at a much lower concentration (0.03 mg/ml sequestrene ~ 0.3 mg/ml BSA), but is less effective on the more sensitive particles from oat coleoptiles.

BSA, since it is commercially available in a crystaline form, was selected as a standard. Figure 7 shows a plot of activity as a function of protein concentration; 0.2 mg/ml appears optimal. The decrease in rate above the optimum is due to foaming during evacuation, which decreases the efficiency of air removal.

ACCURACY AND SPECIFICITY: One of the questions to be considered in assessing the accuracy of an enzyme determination is: to what extent has the enzyme deteriorated prior to or during the time required for estimation? The answer is that, excluding changes caused by cell disruption, there is no deterioration of these two dehydrogenases between maceration and reaction, and their activity is unaffected by considerable variation in the extracting medium.

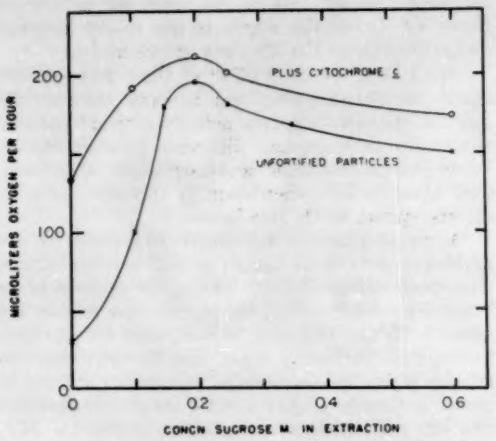


FIG. 8. Effect of sucrose in the extraction medium on the final succinic oxidase activity and its response to cytochrome c. Final reaction mixtures made up to identical sucrose concentrations. Final concentration of cytochrome c: 10^{-4} M.

A valid method must measure activity as proportional to enzyme concentration. This is found to be the case over a wide range with succinic and malic dehydrogenases (fig 5b).

Since the identical particles were used for the study of these two dehydrogenase activities, it is conceivable that each substrate molecule is oxidized more than one step; so that, e.g., the rate of dye decolorization with succinate as substrate was due not only to the dehydrogenation of succinate but of malate as well. However, succinic dehydrogenase was found insensitive to coenzyme I up to a concentration of 2×10^{-4} M, while malic dehydrogenase requires it; hence, with this factor withheld the subsequent steps are prevented. With malic dehydrogenase the subsequent steps are similarly prevented in the usual way by introducing 0.01 M HCN to trap the reaction product.

In the past the effect of malonate has been used for the specific identification of succinic dehydrogenase. Thus, concentrations up to 0.02 M have no effect on malic dehydrogenase; in one experiment the velocity was 0.474 in the absence of malonate and 0.476 in 0.02 M malonate. These concentrations strongly inhibit succinic dehydrogenase (fig 6). The K_I for malonate inhibition of succinate dehydrogenase appears to be 0.0019 M, which represents an affinity some 0.1 that of the substrate for the enzyme. This is remarkable even allowing the interpretation for varying values of K_M/K_I offered by Thorn (40).

MANOMETRIC METHODS: *Extraction:* Unlike the Thunberg tube method, the respirometer requires very specific extraction solutions; in particular, the sucrose concentration is extremely critical. Figure 8 shows that only about 15 % of the optimum oxidase activity was recovered when the tissue was ground in distilled water. The optimum sucrose concentration is about 0.2 M. The lower oxidase activity on either side of 0.2 M appears to be partly related to the availability of endogenous cytochrome c, since the addition of cytochrome c to the reaction mixture at a concentration of 10^{-4} M leads to more nearly uniform rates (fig 8).

The presence of phosphate during extraction exerts a beneficial effect on all 3 oxidase activities. A broad optimum from 0.02 to 0.05 M was found (fig 9).

The addition of protein or Sequestrene to the extracting medium is of little consequence, since abundant soluble pea proteins are present in the homogenate. During further purification, however, a definite effect was shown by these agents. In one experiment succinic oxidase activity was determined after 2 washings of the particles. When BSA at 3 mg/ml or Sequestrene at 1 mg/ml was added to the washing mixture, the succinic oxidase activity was 20 % and 9 %, respectively, greater than the control.

Reaction: There is a further effect of added protein on the linearity of reaction rate (fig 10). A constant rate of O_2 uptake for up to 90 minutes has been recorded in the presence of protein; Sequestrene did not substitute satisfactorily.

The optimal concentration of sucrose for the reaction itself was found to be identical with that for the extraction solution: 0.2 M.

It is not clear whether phosphate is required for O_2 uptake. Although no attempt was made to free the preparation rigorously from phosphate, an absolute requirement with succinic and malic oxidases could not be detected. However, added phosphate does increase the rate (7).

The pH optima are close to that of 7.15 reported

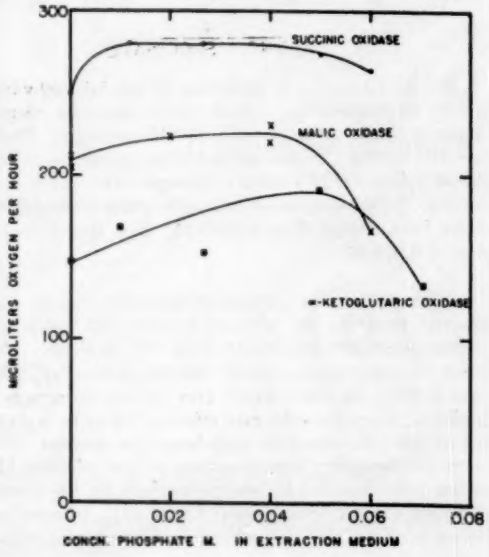


FIG. 9. Effect of phosphate in the extraction medium on the final oxidase activity. With each substrate, concentrations of phosphate in reaction mixture identical.

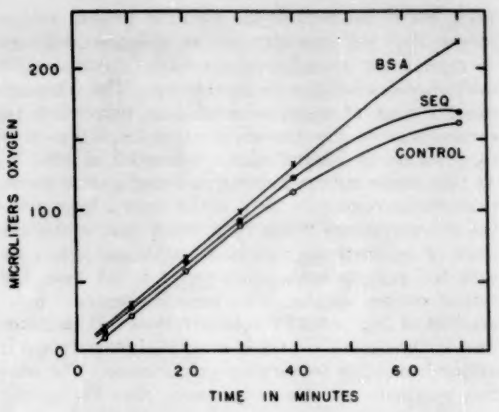


FIG. 10. Effect of protein and Sequestrene on linearity of succinic oxidase activity with time. Thrice-washed particles centrifuged without added protein. BSA or Sequestrene, both 1 mg/ml, added in reaction mixture.

for potato cytochrome oxidase (24), although slight differences are seen amongst the three substrates (fig 11a). The optimum for malic oxidase evidently differs considerably from that for malic dehydrogenase (fig 4).

The phosphate optima for the action of the three oxidases vary from 0.005 M with malic oxidase to 0.04 M with α-ketoglutaric oxidase (fig 11b). In 5 of 6 experiments with succinic oxidase there was a definite phosphate optimum at 0.01 M. This is not simply an osmotic effect since the same value was obtained at several different sucrose and substrate concentrations.

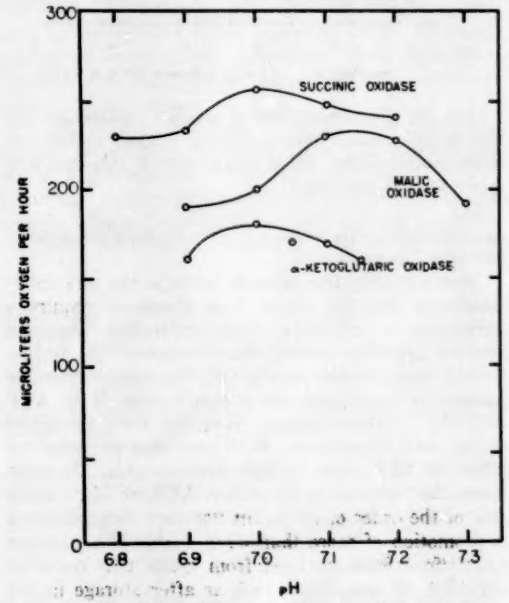
Maximal rates of O_2 uptake were uniformly obtained at about 0.02 M substrate for the 3 oxidases.

Coenzyme I stimulates both α-ketoglutaric oxidase and malic oxidase (table II). With α-ketoglutaric oxidase this promotion is duplicated by ATP. With malic oxidase, in the presence of added coenzyme I, ATP does affect the initial rate of O_2 uptake slightly (table II). The absence of stimulation by ATP alone disagrees with the conclusions of Laties (20). However, added ATP does maintain the linearity of the reaction; in its absence the rate falls off rapidly after 40 minutes, whereas with 3×10^{-3} M ATP, it remains constant for at least 75 minutes. These and other experiments lend themselves to the interpretation that ATP exerts a sparing action on endogenous and added coenzyme I.

Magnesium ion is very clearly a cofactor for α-ketoglutaric oxidase (table III). At 10^{-3} M both O_2 uptake and the disappearance of the substrate are doubled. Malic oxidase is also promoted by magnesium, but to a lesser extent.

The effect of these cofactors on succinic oxidase will be treated in the following section.

Accuracy and specificity: The accuracy of a manometric determination may be judged from the same criteria as were listed for the dehydrogenase-dye system. The linearity of reaction rate has been shown in figure 3. The particles alone do not show exact proportionality between activity and enzyme concentration, but if BSA is added the proportionality becomes clearly linear over a moderate range (fig 12).



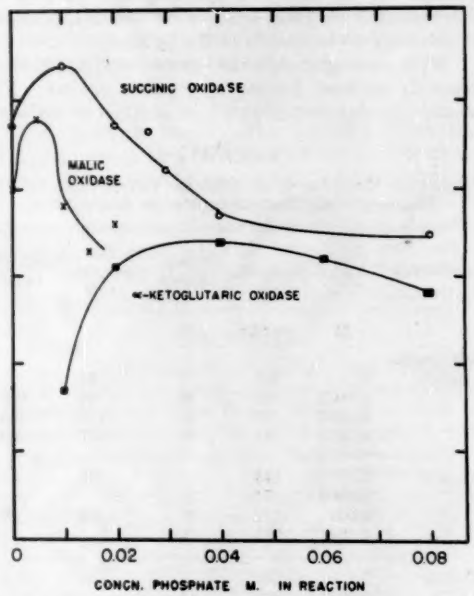


FIG. 11a. Effect of pH on oxidase activity.

FIG. 11b. Effect of phosphate in reaction mixture on oxidase activity. All substrates 0.02 M.

TABLE II

EFFECT OF COENZYME I AND ATP ON RATES OF OXYGEN UPTAKE WITH MALATE AND KETOGLUTARATE. ENZYME RATES CALCULATED OVER LINEAR PORTION OF TIME CURVE

SUBSTRATE	CONC. COENZYME I	CONC. ATP	$-\mu L\ O_2$	STIMULATION
	M	*M*	*per hr*	%
Malate	0	0	108	...
"	0.0001	0	155	43
"	0.0004	0	183	69
"	0.001	0	227	110
Malate	0	0	124	...
"	0	0.001	105	−15
"	0	0.003	86	−33
"	0.001	0	141.5	...
"	0.001	0.0003	189	34
"	0.001	0.001	173	22
"	0.001	0.003	169	19
α-Ketoglutarate	0	0	76	...
"	0.0001	0	128.5	69
"	0.0004	0	141.5	86
"	0.001	0	161	112
α-Ketoglutarate	0	0	76	...
"	0.0004	0.0003	151	99
"	0	0.001	153	101
"	0	0.003	160	111
"	0.0004	0.001	157.5	107

Because the oxidation of any one substrate may proceed by concatenary reactions through the Krebs cycle, the rate of O_2 uptake measured with a complete system like the particles studied here may not be a measure of any single enzyme reaction. On this account the specificity of the enzymes can only be demonstrated by direct analysis for the disappearance of substrate or formation of the product.

With α-ketoglutarate the succinic acid produced is generally oxidized further by succinic oxidase. The second step can be prevented by addition of malonate (27), but it has been found with the present preparations that any concentration of malonate sufficient to repress the second step results in a considerable inhibition of α-ketoglutarate oxidation. The minimum concentration of malonate needed to reduce the O_2 consumption to the theoretical value, i.e., 0.5 mole of O_2 per mole of α-ketoglutarate consumed, is 0.003 M. At this concentration, however, α-ketoglutarate disappearance is reduced by 25 % (31). Over a large number of experiments it has been found that in the absence of malonate the rate of substrate disappearance with this enzyme corresponds to 0.4 to 0.6 times the rate of oxygen uptake. This ratio is essentially independent of Mg^{++} or ATP concentrations. It indicates that in the normal course of events α-ketoglutarate is oxidized smoothly to the stage of fumarate. The relative constancy of this ratio means that O_2 uptake measurements can be used to give a rough estimate of the actual enzyme rate.

TABLE III

EFFECT OF MAGNESIUM ON OXYGEN UPTAKE AND ON THE FORMATION OR DISAPPEARANCE OF KETO ACIDS

SUBSTRATE	CONC. Mg^{++}	$-\mu L\ O_2$	STIMULATION	$\Delta\ \mu L$ α-KETO-ACID	STIMULATION
	M	*per hr*	%		%
α-Ketoglutarate	0	101	..	− 45	...
"	0.0002	200	99	− 96	113
"	0.0005	189	88	−110	145
"	0.0015	181	79	−107	140
Malate	0	143	..	+ 27	...
"	0.0005	174	21	...	...
"	0.001	182	27	+ 34 *	26
"	0.004	165	15	...	...
Malate	0	166	..	...	...
"	0.001	220	32	...	...

* α-Keto acid identified, by absorption spectrum of dinitrophenylhydrazone, as oxalacetate or pyruvate.

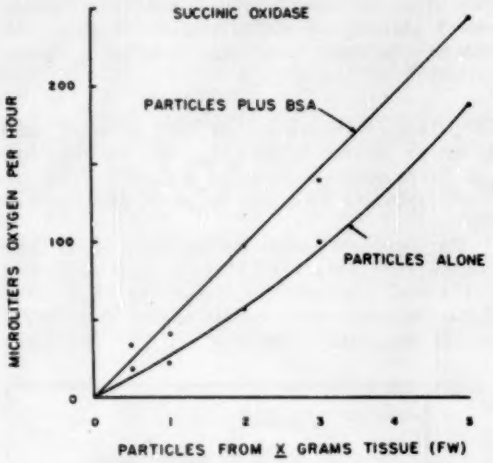


FIG. 12. Proportionality of succinic oxidase activity and enzyme concentration. Linear relation found only when protein added; effect exaggerated in this case with thrice-washed particles.

Succinic dehydrogenase is perhaps the one dehydrogenase that has never been shown to require a coenzyme or cofactor. Data with the Thunberg method (above) confirm this conclusion. It is surprising, then, to find reports (20, 25) that the succinic oxidase of plant particles responds strongly to ATP and Mg^{++}. These findings, however, were confirmed in our own experience. With pea stem particles the effect of ATP alone is slight and variable. In most cases the stimulation by either ATP or Mg^{++} alone was of the order of 10 %, but together they produced a promotion of more than 30 % (table IV). Larger promotions were obtained from tissue that could be regarded as suboptimal, such as after storage in the cold room. By employing such tissue, it was possible to analyze directly for the site of stimulation. The data of table V, obtained from plants stored 24 hours

TABLE IV

EFFECT OF ATP AND Mg^{++} ON OXYGEN UPTAKE WITH SUCCINIC OXIDASE

Conc. Mg^{++}	Conc. ATP	$-\Delta\ \mu L\ O_2$	Stimulation
M	*M*	*per hr*	%
0	0	204	..
0	0.001	206	1
0	0	246	..
0	0.001	272	10
0	0	250	..
0.0015	0	272	9
0	0.001	282	13
0.0015	0.001	342	37

at 3°C, indicates that ATP promotes only the oxidation of malate and further intermediates, and does not affect succinic oxidation per se. On the other hand, Mg^{++} clearly affects the oxidation of succinate itself. With ATP and Mg^{++} together succinate disappearance is not further increased, but O_2 consumption is, and a considerable pool of oxalacetate is formed. From the standpoint of succinic oxidase estimation, it is sufficient to observe that in the absence of cofactors, disappearance of succinate accounts for nearly 90 % of the observed O_2 uptake.

Other experiments bear out the conclusion of table V, that the particles oxidize succinate to fumarate very much more rapidly than to subsequent stages. The R.Q. of succinate oxidation over a 1-hour period was found to be 0.23, which is in accord with a small contribution coming from secondary reactions. That of malate over the same period, on the other hand, was 1.08. In addition, the accumulation of α-keto acid shown in table III is restricted to less than one quarter of the observed O_2 uptake. Both these findings therefore indicate that malate is subject to several secondary oxidations.

It may be said in conclusion that the Thunberg method allows quantitative estimations of succinic and malic dehydrogenases while the manometric method yields less precise, but nonetheless practical assays for succinic and α-ketoglutaric oxidases in these particles.

INTRACELLULAR LOCALIZATION: All of the studies described thus far were conducted on the particulate fraction, M_w, corresponding to the mitochondria of animal tissue. Many dehydrogenases, however, are known to be present in other centrifugal fractions.

TABLE V

EFFECT OF ATP AND Mg^{++} ON OXYGEN UPTAKE AND SUCCINATE DISAPPEARANCE WITH PARTICLES FROM PEA STEMS STORED 24 HOURS AT 3°C

Conc. ATP	Conc. Mg^{++}	$-\Delta\ \mu L\ O_2$	Stimulation	$-\Delta\ \mu L$ succinate	Stimulation	$\Delta\ \mu L$ oxalacetate
M	*M*	*per hr*	%	*per hr*	%	*per hr*
0	0	212	..	188	..	0
0	0	208	..	184	..	0
0.001	0	296	40	186	..	2
0	0.001	333	58	252	35	0
0.001	0.001	380	81	256	38	45

TABLE VI

INTRACELLULAR FRACTIONATION OF SUCCINIC AND MALIC DEHYDROGENASES. CENTRIFUGATION PROCEDURE ACCORDING TO FIGURE 1. ENZYME ACTIVITY EXPRESSED AS CHANGE IN OPTICAL DENSITY PER HOUR PER GRAM FRESH WEIGHT OF TISSUE

Fraction	Succinic dehydrogenase		Malic dehydrogenase	
	v/gm	% of total activity of homogenate	v/gm	% of total activity of homogenate
Homogenate	5.24	100	55.2	100
P_w	0.32	6	3.9	7
S_3	0.56	10.5	41.8	76
M_w	3.88	74	12.5	23
Total	...	90.5 %	...	106 %

In table VI the activities of succinic and malic dehydrogenases in the pea stem fractions are compiled. It is seen that succinic dehydrogenase is almost quantitatively associated with the mitochondria, a finding in agreement with the studies on liver tissue (17). Malic dehydrogenase is present both in particles and in solution. In neither case was there any significant activity in the "plastid" fraction.

The inherent difficulties in studying intracellular localization with the intact cytochrome system are compounded in the case of plant enzymes by their considerable dilution with the vacuolar sap. Nonetheless, a fractionation of succinic oxidase followed manometrically results in complete agreement with the dehydrogenase analysis (table VII).

DISCUSSION

THE ENZYME ASSAY: The modified Thunberg method employed here has many of the qualifications needed for quantitative assay: high sensitivity, specificity, continuous readings, proportionality with time and enzyme concentration, and in the case of malic dehydrogenase, practicality of saturation with coenzyme. The choice of 2,6-dichlorophenolindophenol as a redox dye is essential for plant succinic dehydrogen-

TABLE VII

INTRACELLULAR FRACTIONATION OF SUCCINIC OXIDASE. RATES (v) CORRECTED FOR BLANKS AND EXPRESSED IN μL O_2 PER HOUR PER GRAM FRESH WEIGHT OF TISSUE

Fraction	v	% of total activity of homogenate
Homogenate	97.5	100
P_w	1.2	1.2
M_w	103	105
M_w plus S_3	79	81
M_w plus P_w	102.5	105

ase and is advantageous for malic dehydrogenase. Our experience suggests that the procedures outlined will prove suitable for these enzymes from most plants with only slight alterations.

The neotetrazolium method has been adapted (13) to a demonstration of succinic dehydrogenase in the oat seedling, but the enzyme rate with this oxidant, as calculated from the published data, is no more than 3 % of that with the indophenol dye in the Thunberg method.

The manometric method is not as easy to develop into a quantitative estimation. This is partly due to the properties of the particles. Thus, in Stafford's investigations (38) succinic oxidase, obtained by the centrifugation of homogenates made in water or 0.1 M phosphate, was weak and unstable, the rate decreasing rapidly after 30 minutes of reaction. Less than 60 % of the activity of the original homogenate was recovered in unwashed particles.

The requirement of non-specific protein to maintain stability is not without precedent. Although in the present case no relation was found between phosphate and protein requirements, the suggestions of Keilin and Hartree (18) as to alteration in colloidal properties is clearly applicable. More recently, Sanadi and Littlefield (34) found that BSA promoted the activity of the most purified preparations of liver α-ketoglutaric dehydrogenase.

Another difficulty with the manometric determinations is imposed by the different osmotic and salt requirements of the particles from different plants. The sucrose concentration optimal for pea stem particles (0.2 M) compares closely to the optimum for liver particles (16), but with cauliflower 0.5 M sucrose is necessary (21) and with mung bean (25) the optimum differs between extraction and reaction. The absolute rates of oxidation by mung bean particles suggest that considerable inactivation may have proceeded even under the best conditions described. Although our measurements were conducted at 25°C rather than 30°C, the pea stem particles show Q_{O_2} per mg nitrogen from 300 to 600, or over twice those of the mung bean enzymes for all three substrates.

When a preparation is made under conditions leading to maximum oxidase activity, its activity in the Thunberg tube is not *lower* than maximal. Hence a direct comparison can be made using the factor of 13 μl O_2 per unit of optical density. Thus in one experiment, the $Q_{O_2}{}^N$ for succinate was 395 manometrically and 294 in the Thunberg tube. This agreement is better than that obtained in animal preparations using methylene blue as carrier (37).

Alpha-ketoglutaric oxidase must be estimated under a further limitation, specificity. Approximately 2 atoms of O_2 are absorbed per molecule of keto-acid disappearing even at the beginning. This corresponds to a conversion of α-ketoglutarate to an acid of the oxidation level of fumarate. In the presence of malonate, only 1 atom of O_2 is taken up per molecule of substrate, and the reaction presumably stops at succinate (27). As pointed out above and discussed in detail elsewhere (31), however, it is not possible to suppress the second oxidation without also inhibiting the first. Hence, the assay cannot be conducted with a high degree of accuracy manometrically.

This curious phenomenon of α-ketoglutarate oxidation linked to succinate oxidation is not simply due to the presence of the 2 enzymes on the same particles. With succinate as substrate malate does not begin to be oxidized appreciably until after nearly all of the succinate has disappeared. It would appear, therefore, that the carbon skeleton of α-ketoglutarate remains attached to the particle until after the second oxidation has occurred. Plaut and Plaut (30) have reported similar, but more striking behavior by heart mitochondria; pyruvate is oxidized vigorously via citrate, but citrate itself is not attacked. They reason that the pyruvate condensation and subsequent reactions occur in the mitochondria, but without release of the carbon skeleton until a higher oxidation level is reached.

The above phenomena indicate that manometric assays of complex enzyme systems cannot be interpreted without analysis of the reaction products. There remains, further, the probability that different combinations of linked oxidations may occur with particles from varied sources.

Physical Nature of the Particles: Some of the results reported here bear on the nature and identity of the enzymatic particles. On the basis of staining with Janus Green, and size, Millerd et al (26) identified as mitochondria the fraction centrifuged at 10,000 × g from mung bean hypocotyls. Stafford (38), on the other hand, in a more intensive study of several fractions from germinating pea seedlings, regarded the morphological identification of the particles as unsatisfactory. She writes, "There appears to be a complete series of particles . . . ranging from plastid sizes of 6 to 7 microns down to 0.1 micron or even below." The largest particles are included in her series because a considerable fraction of the cytochrome oxidase and succinoxidase was found therein. This may perhaps have been due to some agglomeration of mitochondria brought about by carrying out the fractionation in water alone. In our experiments (table VII), the entire succinic oxidase activity was recovered in M_w, a fraction which so far as could be seen under the phase microscope does not include units of plastid size and whose homogeneity is directly comparable to liver mitochondria.

Thus, regardless of what future investigations may reveal regarding the identity of the enzymatic particles, the operational analogy between plant particles and animal mitochondria seems clear.

Lastly, some of the above data bear on the statement (25) that the rates of O_2 uptake by particles in vitro are sufficient to account for the respiration of the intact tissue. The actual rate of O_2 uptake with Millerd's best particulate preparation was 45 μl O_2 per hour per gram fresh weight of tissue. The respiration of this tissue when intact was 150 μl per hour. Two factors were employed to equate these values. Firstly, it was stated that only 50 % of the cells appeared to be ruptured during grinding. With our

material, we find, however, a second grinding and extraction of the tissues produces only a small increase in yield. Also when coleoptile tissue had been extracted 3 times, the residue was found to be devoid of succinic dehydrogenase. Secondly, it is claimed that approximately half the mitochondria are lost during the centrifugation process. Table VII shows, however, that with our procedure, which differs from Millerd's only in washing of the plastid fraction, the succinic oxidase activity is quantitatively recovered in M_w. It is believed therefore that the employment of these factors is quite unjustified. This question clearly deserves more detailed consideration, which will be given it elsewhere.

Summary

This paper is concerned with the optimum conditions for the extraction of succinic and malic dehydrogenases as well as of succinic, malic, and α-ketoglutaric oxidases, and with the quantitative assay of these enzymes in plant tissue.

Particles containing the enzymes were prepared by fractional centrifugation from *Avena* coleoptiles and from the apical internodes of etiolated *Pisum* seedlings.

For the dehydrogenases (determined by dye reduction), water was the best extracting medium, but enzyme activity was increased in the presence of a protein preparation, or asparagine, or, to some extent, Sequestrene, during the dehydrogenation; crystalline bovine serum albumin was found very effective. Malic dehydrogenase requires coenzyme I, but succinic dehydrogenase does not; neither enzyme responds to the addition of sucrose or phosphate. As hydrogen acceptor, 2,6-dichlorophenolindophenol was much superior to methylene blue, which is toxic. Succinic dehydrogenase is present only in the particles, but malic dehydrogenase is present also in soluble form, the soluble enzyme accounting for three quarters of the activity of the homogenate.

For the oxidases (determined by O_2 uptake), sucrose (0.2 M) with phosphate (0.02 to 0.05 M) is the optimum extraction medium, and the addition of protein increases activity only of purified preparations. The influence of sucrose is probably exerted on the availability of cytochrome c in the particles. The activity of all 3 enzymes is promoted by magnesium. Details of pH and phosphate optima for the oxidase reactions are given.

Malic and α-ketoglutaric oxidases require coenzyme I; in the latter case, ATP can be substituted, but with malic oxidase ATP has only a slight effect, which is ascribed to its sparing action on coenzyme I. With succinic oxidase an increased O_2 uptake in presence of ATP is shown to be due to the promotion of oxidation processes beyond the stage of fumarate. In the absence of cofactors the disappearance of succinate accounts for nearly 90 % of the observed O_2 uptake.

The limitations to the quantitative recovery of malic and α-ketoglutaric oxidases are discussed, and it is shown that malonate cannot be used to isolate the latter. The dehydrogenases and succinic oxidase are recovered essentially quantitatively by the methods used. Problems involved in comparing the enzyme contents with the respiration of the intact tissue are discussed.

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SEED VITALITY AND OTHER COTTON CHARACTERS AS AFFECTED BY THE AGE OF SEED[1]

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The longevity of a large number of crop or weed seeds has been studied by several workers. Darlington (10, 11) and Toole and Brown (26) reported the results of two experiments, one of which was started by Beal in 1879, and the other by Duvel in 1902: some seed, buried in the soil, kept their vitality for 70 or 60 years respectively. According to other evidence (1, 5), two kinds of seed survived after 149 or 158 years. The life span of seeds has been reviewed by Crocker (9).

In the case of crop plants, seed vitality as affected by age (or various storage conditions) was investigated by Sifton (18), Sonavne (23, 24), Stevens (25), Kiesselbach (13), Robertson and Lute (15), Robertson et al (16, 17), Barton and Garman (4), and others. Alfalfa seed may be viable after 20 years, and the same applies to oats or barley. Wheat also was viable for 15 to 20 years, whereas rye, maize, soybean, timothy, etc. are likely to lose their germinative value within much shorter intervals of time. The life span of crop seeds is greatly affected by the amount of moisture during the period of storage, no seed surviving long when the atmospheric moisture approaches saturation. There is certainly no truth in the belief that in Egypt, wheat seeds have survived from the times of the Pharaohs (14).

Regarding cotton, there is evidence that seeds may still be viable (at the rate of 6%), after being in storage for 25 years under dry conditions; under humid conditions (up to 11% moisture in the seed), they deteriorated rapidly after one to five years according to conditions (19, 20, 21). In the case of higher amounts of moisture, only a few months, weeks, or even days are sufficient for the loss of via-

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bility, depending on the moisture content (7). In California, seed vitality over a 10-year period declined from 84 to 15% (27), while in India, it remained almost unimpaired for about 6 years, then declining very rapidly (24). Besides moisture, temperature was also shown to affect the life span of cotton seed. Both moisture and temperature cannot be high if rapid deterioration is to be avoided; with low temperature cotton seed can withstand high moisture, and vice versa (20, 22). Big-boll varieties, particularly those with large seed, were shown to lose their germinative value easier than small-seed varieties (6). The factors responsible for seed deterioration have been studied by Karon and Altschul (12) and Arndt (2).

The present work was initiated with the view of providing further evidence on the vitality of cotton seed as affected by age; at the same time it aimed at the investigation of possible effects of the age of seed on yield or other characters of economic importance.

Material and Methods

General Considerations: For studying the vitality of seed of different ages, a certain amount of seed was usually set aside; then at yearly or other intervals, representative samples were taken for testing their germinative value. In this way, which was followed by all previous workers in the case of cotton, the same seed was studied in different years, with increasing age; on the other hand, testing conditions were also different, and the study of yield or other crop characters, as affected by age, was made practically impossible.

To obviate these difficulties seeds of the same genetical constitution were produced in successive years; such seeds, obviously differing in age, were then tested in one and the same year.

Experimental Work: From three varieties of the *Gossypium hirsutum* type, seed lots (about 40 lb each), produced in 1942, were set aside for studying the effect of age. This original seed, referred to throughout this paper as S, gave in 1943 rise to two kinds of seed: one by selfing (indicated as A), and the other by open pollination (O). These seeds were tried out in 1944 in two distinct tests, one for comparing seeds S and A of every variety, and the other, seeds A and O. By selfing or open-pollinating, the following kinds of seed were again produced in 1944:

O = from S, by open pollination
A^2 = from A, by selfing
AO = from A, by open pollination
O^2 = from O, by open pollination

The kinds of seed produced since 1942 in every year and for each variety are shown in figure 1 (using the same symbols as above). The same diagram also shows the method of pollination for each type of seed and their ancestry up to the original S. With the seeds available, three types of field tests are every year carried out at the Hellenic Cotton Research Institute, at Sindos. They are known as experiments 9a, 9b, and 9c.

In experiment 9a the seeds of the type S, A, A^2, $A^3 \ldots A^n$ are tested. If selfing does not bring about any appreciable change in the genetical constitution of the seed, this test obviously shows the effect of the age of seed on various properties studied under the same environmental conditions.

In 9b, seeds A^n, $A^{n-1}O \ldots AO^{n-1}$ and O^n (on the right-hand side of the diagonal types A, A, $A^2 \ldots A^n$ in figure 1), were tested, all produced in the same year, namely the n^{th} after 1942. In the same experiment seed O is also included since 1950; this is the original S, sown only once, in the $(n-1)^{th}$ year, after 1942 and open pollinated. If O is about the same as A^n, this obviously means that continuous selfing for n years does not bring about any appreciable change in the genetical constitution of the original seed; thus seeds A, $A^2 \ldots A^n$ may be looked upon as genetically the same but of different age. The same experiment also provides information on the comparative effect of selfing A^n or open pollinating O^n.

In test 9c, seeds O, AO, $A^2O \ldots A^{n-1}O$ (on the left-hand side of the diagonal types S, A, $A^2 \ldots A^n$ in figure 1) are studied, all produced again in the same n^{th} year after 1942 from S, A, $A^2 \ldots A^{n-1}$ seeds by open pollination. If they prove similar, an additional evidence will be obtained that under the conditions of these experiments, selfing for a certain number of generations is unable to bring about noticeable changes in the genetical constitution of the seed; this would also provide an experimental evidence that seeds stored for a different number of years and then all sown for one year, produce seeds practically equivalent.

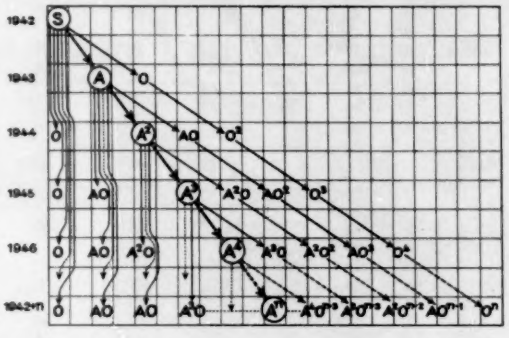

Fig. 1. Diagram showing the types of seed produced every year since 1942 by selfing (A) or open pollinating (O).

In the above way it became possible to study the effect of the age of seed in any year after 1942 with seeds produced in different years, and both in the laboratory and under field conditions. At the same time the correctness of the method was checked by means of the subsidiary tests 9b and 9c.

Seeds S, A, $A^2 \ldots A^n$, for each of the three varieties studied, are always stored at the Cotton Institute; O seeds, produced in different years, are also stored because they may be used instead of A^n, if and when the quantity of the latter will be exhausted, or

differences of any kind would eventually be detected between O and A^n.

METHODS APPLIED: The three *hirsutum* varieties chosen for this experiment were bred from local stock by selection. They are known as 5xa, 6x8, and 16x2, and differ in several crop characters. They were chosen particularly on account of their differences in boll size and the weight of seed (strain 5xa exceeds the other two by far in this respect). All three had been selected in 1932 and selfed for seven generations; for another two years they were open pollinated and then used in this work.

Storage conditions were the same for all types of seed. Placed in burlap sacks they were kept in an ordinary storeroom under natural conditions. However, for avoiding any excessive amount of moisture reaching the seed from the floor of the room, the sacks are hung from the roof. The amount of moisture in the seed varies between 8 and 11 % and their temperature between about 1 and 32°C, according to season.

Besides the germinative value of the seed, the following crop characters have also been studied: yield, earliness, lint length, and ginning out-turn.

TABLE I

MEAN PERCENTAGE OF GERMINATION AS AFFECTED BY AGE

	TYPE OF SEED AND YEAR OF PRODUCTION		YEAR OF TEST							MEAN
			1946	1948	1949	1950	1951	1952	1953	
16x2	S	42	98	98	96	97	97	93	91	95.7
	A	43	98	98	..	98	99	94	97	97.3
	A^2	44	98	92	..	88	86	71	70	84.2
	A^3	45	95	92	92	86	88	81	78	87.4
	A^4	46	..	94	..	89	91	89	92	91.0
	A^5	47	..	92	98	94	93	94	97	94.7
	A^6	48	..	..	96	94	96	92	97	95.0
	A^7	49	..	..	..	91	95	89	96	92.8
	A^8	50	..	..	..	..	88	83	94	88.3
	A^9	51	..	..	..	..	..	96	99	97.5
	A^{10}	52	..	..	..	..	..	..	98	98.0
Mean			97.3	94.3	95.5	92.1	92.6	88.2	91.7	92.2
D. s. s.*			± 5.0	± 3.6	± 3.6	± 4.8	± 4.8	± 5.0	± 3.4	
6x8	S	42	94	96	95	87	97	86	83	91.1
	A	43	100	94	..	91	96	87	87	92.5
	A^2	44	98	90	..	84	88	64	70	82.3
	A^3	45	78	82	85	72	86	59	60	74.6
	A^4	46	..	96	..	91	92	84	91	90.8
	A^5	47	..	88	95	87	86	81	96	88.8
	A^6	48	..	..	99	93	98	90	98	95.6
	A^7	49	..	..	..	86	92	85	97	90.0
	A^8	50	..	..	..	..	94	89	98	93.7
	A^9	51	..	..	..	..	..	71	94	82.5
	A^{10}	52	..	..	..	..	..	..	96	96.0
Mean			92.5	91.0	93.5	86.4	92.1	79.6	88.2	88.0
D. s. s.*			± 5.0	± 3.6	± 3.6	± 6.3	± 4.8	± 5.0	± 4.4	
5xa	S	42	97	82	93	84	90	80	72	85.4
	A	43	94	88	..	86	93	79	77	86.2
	A^2	44	95	94	..	93	89	76	77	87.3
	A^3	45	88	90	92	86	89	78	80	86.1
	A^4	46	..	96	..	89	93	87	87	90.4
	A^5	47	..	88	93	89	90	89	93	90.3
	A^6	48	..	..	97	91	95	94	91	93.6
	A^7	49	..	..	..	66	90	73	91	80.0
	A^8	50	..	..	..	..	89	85	92	88.7
	A^9	51	..	..	..	..	..	77	88	82.5
	A^{10}	52	..	..	..	..	..	..	91	91.0
Mean			93.5	89.7	93.8	85.5	90.9	81.8	85.4	87.4
D. s. s.*			± 5.0	± 3.6	± 3.6	± 6.9	± 4.8	± 6.7	± 5.6	
n **			33	40	45	35	32	45	70	
r †			4	9	6	6	6	6	8	

* Difference statistically significant at the 5 % level for values within varieties in every year of test. It is equal to $t2\sqrt{\frac{s^2}{r}}$ where t is the value from the appropriate tables, s^2 stands for mean square, and r for the number of replications, as usual.

** Number of degrees of freedom corresponding to error.

† Number of complete replications.

All four crop characters were determined for every plot separately. Yield is expressed in kg of seed-cotton per 1000 m², while for earliness the mean date of maturity, as described by Christidis and Harrison (8), is made use of. Lint length represents average length in mm; it was obtained by means of the Ball's sorter (3), using only the draw box for making a uniform sliver.

After 1949, when the types of seed of different age became quite numerous, only a few intermediate types (not the same every year) were included in each experiment, together with the first and the last type of the series.

Results

Seed Vitality: The mean percentage of germination for seeds of different age, as obtained in various years, is shown in table I. Proper germination tests were not always possible during or immediately after the last world war and that is why some data are missing.

Obviously the percentage of germination varies considerably, not only for seeds produced in different years, but also for the same seed when tested in different years. Seed S of variety 6x8, for instance, when tested in various years, gave the following percentages of germination:

Year of test	Age of seed (in years)	Germination %
1946	4	94
1948	6	96
1949	7	95
1950	8	87
1951	9	97
1952	10	86
1953	11	83

Table II

Mean Yields (Kg of Seed Cotton per 100 m²) of Different Age Groups, as Obtained by All 9a Experiments

Type of seed and year of production			Year of test									Mean
			1944	1945	1946	1947	1948	1949	1950	1951	1952	
16x2	S	42	109	181	300	360	183	145	186	120	275	206.6
	A	43	110	185	281	352	194	134	...	...	...	209.3
	A²	44	...	224	278	279	194	139	...	139	...	208.8
	A³	45	...	...	274	290	197	135	194	...	291	230.3
	A⁴	46	...	...	...	301	196	147	...	...	...	181.3
	A⁵	47	...	...	...	...	184	142	...	160	...	160.0
	A⁶	48	...	...	...	...	...	139	208	...	302	216.3
	A⁷	49	...	...	...	...	...	...	199	...	...	199.0
	A⁸	50	...	...	...	...	...	...	...	147	...	147.0
	A⁹	51	...	...	...	...	...	...	...	...	300	300.0
Mean			109.5	196.7	283.3	316.4	191.3	140.1	196.8	141.5	292.0	
6x8	S	42	116	238	316	207	187	105	176	105	261	190.1
	A	43	105	238	293	334	178	125	...	...	...	212.2
	A²	44	...	226	186	205	177	112	...	102	...	168.0
	A³	45	...	...	247	223	188	129	184	...	250	203.5
	A⁴	46	...	...	...	282	185	119	...	...	...	195.3
	A⁵	47	...	...	...	...	193	118	...	123	...	144.7
	A⁶	48	...	...	...	...	...	105	186	...	291	194.0
	A⁷	49	...	...	...	...	...	...	191	...	...	191.0
	A⁸	50	...	...	...	...	...	...	...	124	...	124.0
	A⁹	51	...	...	...	...	...	...	...	...	244	244.0
Mean			110.5	234.0	260.5	250.2	184.7	116.1	184.3	113.5	261.5	
5xa	S	42	111	230	258	138	174	114	161	81	237	167.1
	A	43	103	252	187	280	181	117	...	...	...	186.7
	A²	44	...	192	242	266	190	119	...	103	...	185.3
	A³	45	...	...	231	340	203	122	171	...	254	220.2
	A⁴	46	...	...	...	305	184	122	...	...	...	203.7
	A⁵	47	...	...	...	...	188	118	...	112	...	139.3
	A⁶	48	...	...	...	...	...	126	182	...	278	195.3
	A⁷	49	...	...	...	...	...	...	160	...	...	160.0
	A⁸	50	...	...	...	...	...	...	...	117	...	117.0
	A⁹	51	...	...	...	...	...	...	...	...	289	289.0
Mean			107.0	224.7	229.5	265.8	186.7	119.7	168.5	103.3	264.5	
D. s. s.*			n.s.	± 33.6	± 52.7	± 88.7	n.s.	n.s.	n.s.	± 20.2	± 26.0	
n**			11	18	27	36	75	90	63	63	63	
r†			5	4	4	4	6	6	8	8	8	

* Difference statistically significant at the 5 % level, for values within varieties in every year of test.
** Number of degrees of freedom corresponding to error.
† Number of complete replications.

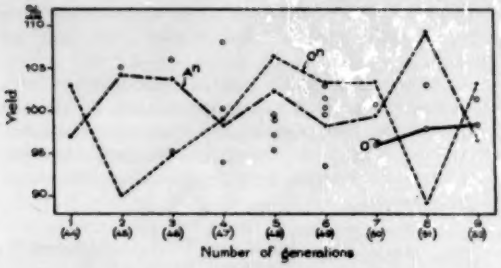

FIG. 2. The effect of selfing (A^n) or open pollinating (O^n) for up to 9 generations on yield (percentage of variety and year means). Data from experiments 9b; averages for all three varieties (numbers in parenthesis denote the year of test, while the small circles represent the yield of seeds intermediate in the method of pollination between A^n and O^n).

Rise or fall in the percentage of germination is often erratic showing that, besides the effect of age, testing conditions or sampling errors may also be responsible for the differences observed.

On the other hand seeds produced in certain years proved always better or worse than others, as may be seen from the data of variety 6x8, referring to seeds S and A^3. In all seven experiments the latter type of seed showed invariably a lower percentage of germination than the former (94—78, 96—82, 95—85, 87—72, 97—86, 86—59, 83—60), the same conclusion being reached when the comparison is made between data obtained in different years but referring to seeds of the same age (94—85, 96—86, 95—59, 87—60). Therefore, besides sampling errors (which every step was taken to reduce as much as possible), and the effect of age, the data in table I seem to be affected also by the conditions of each test, as well as by those prevailing during the year the respective seeds were produced.

It is worth noticing that seed produced in some years deteriorates more rapidly than seed produced in others. With variety 16x2 for example, seeds A^2 and A^3 show a reduced percentage of germination after four or five years, although other seed, even older, still keeps its vitality unimpaired after 11 years. The same applies to seed A^2 of variety 6x8.

The data in table I indicate that so far as germination is concerned, mean values for seeds produced in different years are more variable than mean values of the yearly tests. As it will be seen later, the opposite is the case with yield.

YIELD: All yield data so far obtained from experiments 9a (mean values for every variety and age group in each test), are given in table II.

Until 1950 included, seeds of different age yielded about the same results; the significant results in 1945–47 are inconsistent and obviously due to a few erratic values. In 1951 and 1952, however, variety 5xa showed a small but unmistakable reduction in yield with the older seed, the same, although to a smaller degree, also applying to variety 16x2. In the case of 6x8 there was not any consistent effect.

These conclusions do not necessarily hold for seeds of different age, unless seeds S, A, $A^2 \ldots A^n$ prove genetically equivalent. Seven 9b experiments (from 1944 to 1950), showed unequivocally that selfing or open-pollinating produces seed of the same yielding ability. These seeds were also shown equivalent to O, which is the original S seed open pollinated only once in the $(n-1)$th year. In 1951, the results proved statistically significant; however, they were inconsistent and far from establishing any difference between A^n and O^n, or between these two and O seed (fig 2). To similar conclusions one is also led from the data of experiments 9c. They show further that seeds O, AO, $A^2O \ldots A^{n-1}O$, remaining in storage for a different number of years (before being open pollinated in the year previous to that of test), were practically equivalent (fig 3).

Under the conditions of these experiments it is shown that selfing, up to 9 generations at least, does not seem to affect the productivity of cotton seed; selfed seeds do not differ significantly from seeds produced by open pollination, and both types seem equivalent to O seed. In consequence, so far as yield is concerned, the classes of seed in experiments 9a are genetically similar to the original S seed and any difference between them may rightly be attributed to age (or to conditions in the year the respective seed was produced).

EARLINESS: This character has been studied in all field experiments since 1945. The effect of age on earliness was found significant in only two 9a experiments (out of seven); that of the method of pollination, in one 9b experiment (also out of seven), whereas that of the number of selfed generations, in two 9c experiments (out of six). These significant results are all illustrated in figure 4.

Even in the above five tests, which proved to be statistically significant, the results are inconclusive (as may be seen by comparing tests 1948a and 1951a, or 1948c and 1950c, in figure 4). At any rate the differ-

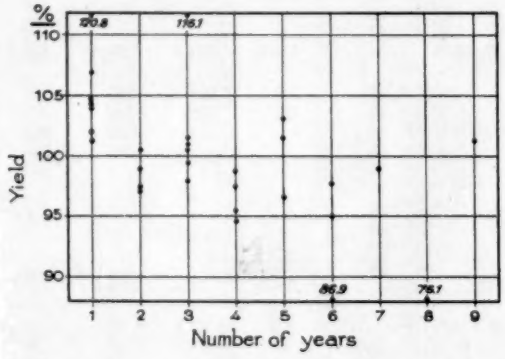

FIG. 3. Yield (percentage of variety and year means) as affected by the number of years seeds O, AO, $A^2O \ldots A^{n-1}O$ remained in storage, before open pollinated in the year previous to that of test.

ences observed were exceedingly small, and in 15 other experiments proved to be entirely negligible. In view of this evidence it is safe to conclude that, as judged by earliness, seeds S, A, $A^2 \ldots A^n$ are genetically the same, and earliness of maturity is not affected by the age of seed.

GINNING OUT-TURN: The data of ginning out-turn are similar to those referring to earliness. In spite of one or two values which were statistically significant but very inconsistent, all the evidence points to the conclusion that seed age does not affect the percentage of lint; also that, according to data by experiments 9b and 9c, no genetical differences can be assessed in this respect to seeds S, A, $A^2 \ldots A^n$.

LINT LENGTH: The same applies to lint length which, on the evidence obtained, does not seem to vary according to either age or method of pollination.

DISCUSSION

SEED VITALITY: The individual tests in table I are subject to considerable variation, which is not all random or attributable to age; a large part of it is also due to the unmistakable fact that seeds produced in different years are not alike, at least as judged by their rate of germination.

The effect of age, although always statistically significant in the individual tests, seems mostly erratic on account of the concomitant "year of production" effects just mentioned. There hardly appears to be any consistent drop (or otherwise) in vitality with increasing age. In the case of variety 16x2, vitality seems to be beautifully maintained for at least 11 years, only seed produced in 1944 and 1945 showing distinct signs of deterioration. The same applies to variety 6x8; here, however, seed 9 or 10 years old, although still germinating at a rate higher than 80 %, does not exceed the 90 % mark, as is the case with 16x2. Finally, in 1953, the big-seed variety, 5xa, showed a marked reduction in vitality with age.

In this connection it would be most advisable to combine the evidence from all experiments in table I, but this meets with great difficulty. Treatments (age) available in successive years are necessarily different, so that part of "age" effects is confounded with the effect of "year of test." The same applies to seed produced in different years (S, A, $A^2 \ldots A^n$), the quality of which is not always the same. Therefore, "age," "year of test," and "year of production" effects are to a certain extent confounded with one another so that the separate effect of each cannot be assessed with certainty. The same difficulty is faced when studying the effect of age with the same seed in different years; this effect is again confounded with that of "year of test."

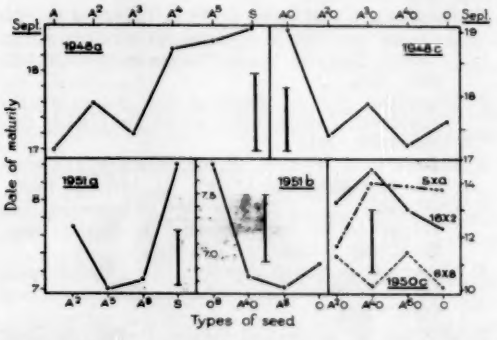

FIG. 4. Earliness as affected by the age of seed and kind of pollination (the small rods denote the difference needed for significance, while a, b, or c, after the year, show whether the respective experiment was 9a, 9b, or 9c).

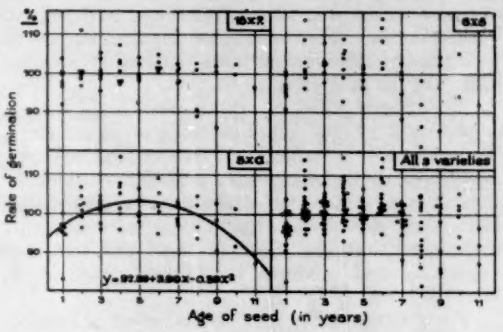

FIG. 5. Rate of germination as affected by age (percentages of year of test and year of production means).

To provide an illustration of the general effect of "age" on seed vitality, based on all tests in table I, the target diagrams shown in figure 5 have been worked out. Such diagrams may be based on the actual figures in table I, or on values corrected for "year of test," "year of production" or both (by expressing the original figures as percentages of "year of test" and "year of production" means). One could also express germination as percentage of the one-year-old seed which is included in every experiment. All these methods have been tested and finally values corrected for both "year of test" and "year of production" were chosen for figure 5, since they are the only ones showing some relationship between age and seed vitality. The regression of seed age on the rate of germination for variety 5xa proved to be highly significant, so that a second order polynomial could perfectly be fitted to the data (fig 5); this, however, was not the case with variety 16x2 or 6x8.

The results indicate that the rate of germination increases during the first five years of storage, and then gradually decreases; however, even after 11 years the drop is very small to be of any practical consequence. Data in subsequent years will show whether this is definitely so, or whether these results are due to the small number of tests with the older seed or to the interference of the confounding already referred to. The only other instances in which cotton seed remained perfectly viable after more than 10 years in storage (up to 15 or 20 years under certain conditions), are reported by Simpson (21, 22).

YIELD: From the data in table II it is obvious that, as it should be expected, tests carried out in different years (and on different fields) show great variation in

average yield, and this variation is much greater than that due to the year the seed has been produced. It seems that any effect of environmental conditions on seed quality affects much more the rate of germination than the final yield.

The general effect of age on yield could best be brought out by the pooled evidence of all individual 9a experiments in table II. However, the statistical analysis of the respective data is subject to the same difficulties as in the case of the data on germination. Here again part of the "age" effects are inextricably confounded with the "year of test," and the same applies to seed produced in different years (S, A, $A^2 \ldots A^n$).

For eliminating the very marked effect of "year of test," the values in table II were first expressed as percentages of the variety means in each experiment; then the target diagrams in figure 6 were worked out, where percentages of the same age are grouped together. In spite of the obvious trend (with variety 5xa) for older seed to yield less, an attempt to fit

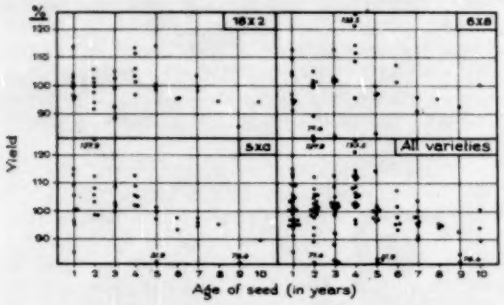

FIG. 6. Yield (in percentage of "year of test" means) as affected by the age of seed.

straight lines or curves to the data in figure 6, showed that regression between yield and age of seed was never statistically significant. Therefore, on the light of these experiments, seed up to 10 years old yields about the same as fresh seed. Results in the next few years will show whether the small decline in yield, observed particularly with variety 5xa, represents actually the effect of age or may be attributed to chance (the number of tests including the older seeds was necessarily very limited).

SUMMARY

1. In 1942 specific work was started at the Hellenic Cotton Research Institute (at Sindos) for studying the effect of the age of seed on germination, yield, and other crop characters. Three Upland cotton varieties are used in this respect.

2. Laboratory and field tests were carried out in every year, with seed produced in successive years by selfing. Subsidiary experiments showed that in this way the genetical constitution of the seed remained practically unchanged.

3. Great variability was noticed in the data of all the characters under investigation. The results were affected not only by the usual errors of sampling or by the age of seed, but also by the conditions of test or those prevailing at the time the respective seed was produced.

4. On the whole the seed of all three varieties germinated quite satisfactorily after being in storage for 11 years. However, the big-seed variety 5xa showed a marked decline, whereas 16x2 still germinates at the rate of over 90 %.

5. Regarding yield, the effect of age seems rather negligible for the first 10 years of storage, although in the case of 5xa there is some indication that yield decreases with age (after the 5th or 6th year). So far as earliness, lint length, or ginning out-turn are concerned, age does not seem to have any consistent effect on them.

6. As judged by yield, earliness, lint length, or ginning out-turn, selfing for at least nine generations does not seem to affect the genetical constitution of the seed.

7. The great variability of the results obtained indicates that isolated tests are not of much value in studying the effect of the age of seed. Proper experiments carried out for a large number of years and with seed produced under different conditions are necessary.

8. The combined statistical analysis of all the data of long term experiments, such as those dealt with in this paper, meets with great difficulty on account of an unavoidable partial confounding of the effects of age, year the seed is produced, and year of test. Special methods of analysis may be needed in this respect.

9. The work is continuing until the seed will completely deteriorate with age.

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VITAMIN CONTENT OF TURNIP-GREEN PLANTS IN RELATION TO GROWTH[1, 2]

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Correlations between vitamin concentration and dry weight in certain plant organs have been noted by various investigators. Wynd (8) observed that an increased concentration of carotene in the leaves of oats and rye harvested at the jointing stage was associated with increased yields of dry matter, but that concentration of ascorbic acid was not intrinsically related to yield. Also working with oat plants, Watson and Noggle (7) found that riboflavin concentration in leaves and stems, in μg per gm, was significantly correlated (5 % level) with the growth of these organs (dry weight per plant), but that correlation of riboflavin concentration with the growth of roots was not significant.

The present experiments with turnip-green plants were carried out in connection with a regional project. They were undertaken to determine the concentration of vitamins in all organs and the distribution of vitamins among organs as plant dry weight increased, and to study the relationship between the total amount of vitamin per plant and plant dry weight.

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[3] The experimental data in this paper are taken from a thesis submitted by George V. Odell in partial fulfillment of the requirements for the degree of Master of Science in Biological Chemistry in the Graduate School of the Oklahoma A. and M. College and were presented, in part, at the spring, 1952, meeting of the American Chemical Society at Milwaukee, Wis.

Materials and Methods

Production of Crops: Three experiments were conducted with field-grown turnip-green plants of the Seven-Top variety (*Brassica rapa* L.); plants in the first experiment were grown at Stillwater in the fall of 1950, and those in the second and third experiments were grown at Stillwater and Perkins in the spring of 1951. Seed was planted in six 200-foot rows two and one half feet apart, and a complete fertilizer was applied as a side band application to each row at the time of planting. To facilitate sampling, each row was divided into ten-foot segments.

Duplicate samples were taken at every harvest. A sample consisted of an equal number of plants from each of five segments; each segment was in a different one fifth of the field and was randomly selected from the 24 segments in that area. All segments were sampled from the same end and plants were taken in order of their occurrence. A minimum of ten plants was used for each sample and these were always the first two plants in each segment having a plant grow-

TABLE I

MEAN * VITAMIN CONTENT OF LEAF BLADES, MIDRIBS, AND ROOTS OF TURNIP-GREEN PLANTS (SEVEN TOP) FROM THREE SOURCES

LOCATION AND SEASON	AMOUNT OF VITAMIN PER GRAM DRY MATTER										
	ASCORBIC ACID MG			RIBOFLAVIN μG			THIAMINE μG			CAROTENE MG	
	LB †	M †	R †	LB	M	R	LB	M	R	LB	M
Stillwater, fall	10.6	7.7	8.0	28	8	7	13	3	5	0.51	0.05
Stillwater, spring	10.0	8.2	8.3	29	8	8	14	4	7	0.48	0.04
Perkins, spring	10.2	7.5	7.3	26	7	7	12	2	5	0.48	0.04
Mean ** of combined expts.	10.3	7.8	7.8	28	7.8	7	13	3	6	0.49	0.04
Standard deviation	±1.4	±1.4	±1.4	±4	±2	±1.4	±4	±1.6	±2.9	±0.10	±0.04

* Mean of 24 values for duplicate field samples taken on 12 sampling days over a period of six weeks.
** Mean of 72 values.
† LB = leaf blades; M = midribs; R = roots.

ing on either side of them in the row. If more than ten plants were required, more than two were taken from each segment.

PREPARATION OF SAMPLES: Sampling began five weeks after planting and was continued for six weeks, samples being collected at 8 a.m. on two fixed days each week. Plants were pulled or dug from the row and taken at once to the laboratory where the roots were removed and the leaves separated. Leaves and roots were washed in tap water, rinsed twice in distilled water and allowed to drain. Midribs were carefully removed from the leaves and the fresh weight of leaf blades, midribs, and roots determined. Each leaf-blade, midrib, and root composite was mixed, diced, and analyzed for moisture, ascorbic acid, riboflavin, and thiamine. Carotene was determined in leaf-blade and midrib composites but analysis of root composites for this constituent was discontinued after it was established that there was no carotene present in this organ.

ANALYTICAL PROCEDURES: *Moisture:* Moisture content was determined by drying duplicate 50 gm samples of the fresh diced material to constant weight in a forced-draft oven at 70°C.

Ascorbic acid: Ascorbic acid was determined by the photometric method developed by Heinze et al (2).

Riboflavin: A modification of the procedure described by Peterson et al (4) was employed in the determination of riboflavin. Details of the method as used in this study have been described by the Southern Cooperative Group (6, pp. 138–139).

Thiamine: The procedure used for the determination of thiamine was an adaption of the method developed by Conner and Straub (1) as described by the Southern Cooperative Group (6, pp. 134–138).

Carotene: Carotene was determined by the procedure developed by Moore and Ely (3).

Methods used in the analysis of variance and in correlation and regression studies were those described by Snedecor (5).

RESULTS AND DISCUSSION

VITAMIN CONCENTRATION IN LEAF BLADES, MIDRIBS, AND ROOTS: Vitamin concentration in the organs of plants from the different sources was strikingly consistent (table I). The mean amount of each of the vitamins per gram dry matter in leaf blades did not vary significantly among the three experiments. Nor was there significant variation in the mean vitamin content of midribs and roots from the different sources, except that in one experiment the thiamine content of midribs and the riboflavin content of roots were slightly higher than in the other two experiments.

Concentration of ascorbic acid and thiamine in leaf

TABLE II

MEAN * PERCENTAGE OF THE TOTAL AMOUNT OF DRY MATTER AND VITAMIN PER PLANT CONTAINED IN THE LEAF BLADES, MIDRIBS, AND ROOTS OF TURNIP-GREEN PLANTS (SEVEN TOP) FROM THREE SOURCES

LOCATION AND SEASON	PERCENTAGE OF TOTAL AMOUNT OF CONSTITUENT IN PLANT													
	DRY MATTER			ASCORBIC ACID			RIBOFLAVIN			THIAMINE			CAROTENE	
	LB †	M †	R †	LB	M	R	LB	M	R	LB	M	R	LB	M
Stillwater, fall	62	28	10	69	23	8	86	11	3	87	7	6	96	4
Stillwater, spring	57	32	11	62	28	10	81	14	5	80	12	8	96	4
Perkins, spring	57	30	13	65	25	10	83	12	5	84	7	9	96	4
Mean ** of combined expts.	59	30	11	66	26	9	83	12	4	84	9	7	96	4
Standard deviation	±6	±5	±4	±8	±6	±2	±5	±4	±2	±8	±6	±4	±2	±2

* Mean of 24 values for duplicate field samples taken on 12 sampling days over a period of six weeks.
** Mean of 72 values.
† LB = leaf blade; M = midrib; R = root.

blades remained practically unchanged throughout the experimental period. With these exceptions, concentration of each of the vitamins in the different organs decreased as plant weight increased. In the combined experiments, correlation coefficients relating vitamin concentration and the dry weight of the organ per plant were negative and highly significant (1 % level) for carotene in leaf blades (−0.578), and midribs (−0.754); for riboflavin in leaf blades (−0.516), midribs (−0.607), and roots (−0.376); and for ascorbic acid in midribs (−0.522), and roots (−0.671). Thiamine concentration in the midribs was significantly (5 % level) correlated (−0.278) with the dry weight of this organ, but in the roots the correlation was not significant.

Distribution of the Whole Plant Content of Dry Matter and Vitamins among Leaf Blades, Midribs, and Roots: Distribution of the whole plant content of dry matter and vitamin among plant organs (table II) was consistent in the three experiments. The greatest proportion of each vitamin was always found in the leaf blades, which contained about 96 % of the plant carotene and 84 % of the total thiamine and riboflavin content. Distribution of ascorbic acid varied more than that of any of the other vitamins and followed the distribution of dry matter rather closely. In a period of six weeks, the percentage of total plant dry matter decreased significantly (1 % level) from 75 to 45 % in leaf blades, and increased significantly (1 % level) from 23 to 39 % in midribs, and from 5 to 15 % in roots. Similarly, the percentage of total plant ascorbic acid decreased significantly (5 % level) from 73 to 58 % in leaf blades, and increased significantly (5 % level) from 21 to 32 % in midribs, and from 6 to 10 % in roots.

Relationship between Amount of Vitamin per Plant and Plant Dry Weight: Growth, as measured by plant dry weight, was not uniform in the three experiments (table II); the mean weight of plants grown at Perkins was significantly less (1 % level) than that of plants in the other experiments. In the combined experiments, the maximum mean dry plant weight attained was 18.5 times the mean weight at the initial sampling, and the mean amount of ascorbic acid, riboflavin, thiamine, and carotene per plant increased 14-, 11-, 15-, and 9-fold, respectively.

The total amount of each vitamin per plant was positively and significantly (1 % level) correlated with the total amount of dry matter per plant in each experiment. The relationship between the total amount of vitamin and the plant dry matter was maintained regardless of plant weight, since all partial correlation coefficients determined with the age of plant held as a constant were highly significant.

The lowest partial correlation coefficients, those for carotene and riboflavin, were the result of a highly significant (1 % level), progressive decrease in the amount of each of these vitamins per gram dry matter of the whole plant (fig 1). In the combined experiments carotene decreased from 0.43 mg to 0.19 mg, and riboflavin from 26 μg to 14 μg per gm dry matter in whole plant during the six weeks' period. The amount of ascorbic acid per gm dry plant weight also decreased significantly (1 % level), but the decrease was less than in the case of carotene and riboflavin. There was no significant variation in the amount of thiamine per gm dry matter of whole plant. The mean and standard deviation of the amount of each vitamin per gram dry matter in the combined experiments were: ascorbic acid, 9 ± 1.3 mg; riboflavin, 10 ± 2.6 μg; thiamine, 9 ± 2.4 μg; and carotene, 0.3 ± 0.05 mg. Decrease in the amount of vitamin per gm dry matter of whole plant as plant weight increased was largely the result of an increasing percentage of the total plant dry matter in midribs and roots.

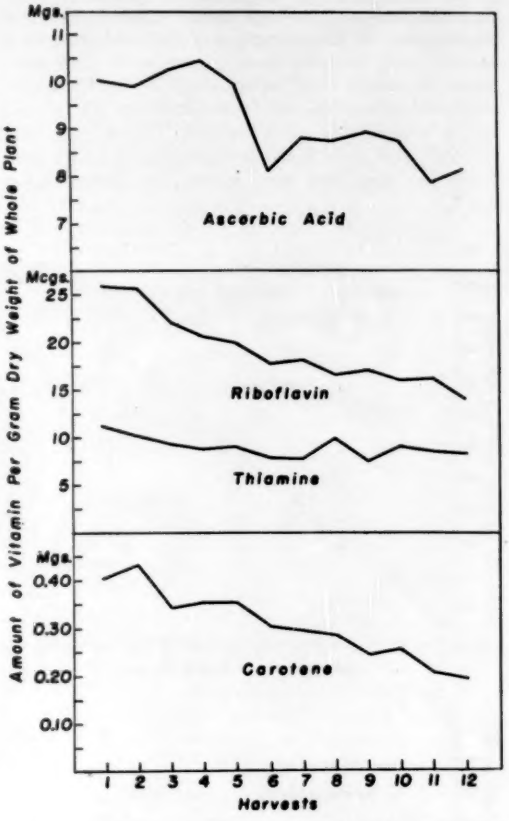

Fig. 1. Mean amount of vitamin per gram total plant dry weight in turnip-green plants (Seven Top) at twelve harvests over a period of six weeks.

Regression coefficients to determine the increase in the total amount of ascorbic acid in the plant per unit increase in plant dry weight were calculated for the individual experiments, and an analysis of errors of estimate showed no significant variation among the three coefficients. Differences in the environmental conditions under which the plants were produced failed to affect the relationship between the amount

of ascorbic acid in the whole plant and the dry weight of the plant. Similarly, regression coefficients for the amount of thiamine per plant on plant dry weight did not vary significantly among experiments, nor did regression coefficients for the amount of carotene per plant on plant dry weight. There was a significant variation (5% level) among the three regression coefficients for riboflavin; the rate of increase in plant riboflavin content in the experiment at Perkins differed slightly from the rate in the other experiments.

On the assumption of a linear relationship, regression equations were derived from the data in the three experiments for the estimation of the total amount of ascorbic acid, thiamine, and carotene in the plant from plant dry weight; and an equation for the estimation of riboflavin was derived from data from the fall and spring experiments at Stillwater. These equations, the regression lines, and the experimental values from which the equations were derived are shown in figure 2.

The derived equations adequately express the relationship between the vitamin content of the whole plant and the plant dry weight when plant weight varied from 2 grams to 69 grams. Since these equations were applicable to plants produced at different locations and in different seasons, it appears probable that they may apply to turnip-green plants of the Seven Top variety regardless of their source. It is even possible that they are applicable to all varieties of turnip-green plants. Use of this type of equation provides a convenient method for the estimation of the vitamin content of the plant from plant dry weight.

Summary and Conclusions

Vitamin concentration in leaf blades, midribs, and roots of turnip-green plants from three sources appeared to be characteristic for the variety, and relatively unaffected by environmental conditions. As plant weight increased, variation in the ascorbic acid,

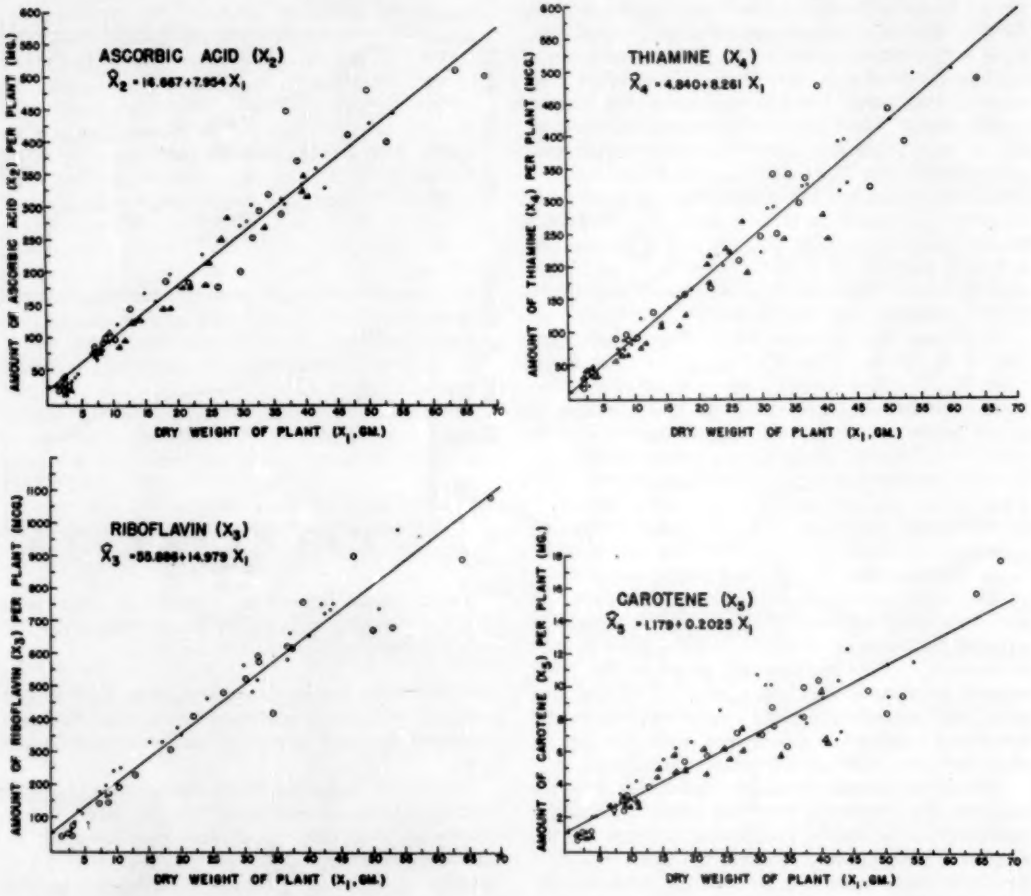
ASCORBIC ACID (X_2)
$\hat{X}_2$ = 16.667+7.954 X_1
AMOUNT OF ASCORBIC ACID (X_2) PER PLANT (MG.)
DRY WEIGHT OF PLANT (X_1, GM.)
THIAMINE (X_4)
$\hat{X}_4$ = 4.840+8.261 X_1
AMOUNT OF THIAMINE (X_4) PER PLANT (MCG.)
DRY WEIGHT OF PLANT (X_1, GM.)
RIBOFLAVIN (X_3)
$\hat{X}_3$ = 58.886+14.979 X_1
AMOUNT OF RIBOFLAVIN (X_3) PER PLANT (MCG.)
DRY WEIGHT OF PLANT (X_1, GM.)
CAROTENE (X_5)
$\hat{X}_5$ = 1.179+0.2025 X_1
AMOUNT OF CAROTENE (X_5) PER PLANT (MG.)
DRY WEIGHT OF PLANT (X_1, GM.)

FIG. 2. Regression of amount of vitamin per plant on dry plant weight in turnip-green plants (Seven Top). Stillwater, fall = △; Stillwater, spring = ●; Perkins, spring = ○.

riboflavin, thiamine, and carotene content of leaf blades was not statistically significant over a period of six weeks. In midribs and roots, the concentration of each of the vitamins decreased as the plants grew larger and the decrease was statistically significant for each vitamin except carotene, in midribs, and thiamine, in roots.

Distribution of the whole plant content of carotene, riboflavin, and thiamine among plant organs did not change appreciably as plant dry weight increased, but there was a marked change in the distribution of ascorbic acid which rather closely followed the distribution of plant dry matter.

In all experiments the total amount of each vitamin per plant was positively and significantly correlated with the total amount of plant dry matter. Increase in the total amount of vitamin per plant per unit increase in plant dry matter was characteristic for each vitamin and was not influenced by the environmental conditions under which the plants were grown. Regression equations derived for the estimation of the amount of each of the vitamins per plant from plant dry weight were applicable to plants weighing from 2 grams to 69 grams.

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THE EFFECT OF TEMPERATURE ON THE CONVERSION OF PROTOCHLOROPHYLL TO CHLOROPHYLL A IN ETIOLATED BARLEY LEAVES[1]

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Protochlorophyll is converted to chlorophyll when dark-grown leaves containing it are illuminated, but it is not converted to chlorophyll when dissolved in organic solvents and illuminated. Conditions exist in the leaf, therefore, which facilitate this conversion. In order to analyze the mechanism of the transformation, the effects of various factors on the reaction have been examined. Among the factors examined is temperature, and it is chiefly with this factor that this paper deals.

Only fragmentary data concerning the effect of temperature on this conversion have been published. Liro (12) found that irradiation of etiolated seedlings at −15°C produced chlorophyll. Scharfnagel (15) detected chlorophyll formation in dark-grown corn seedlings when they were illuminated at −6°C. Lubimenko (13) observed that temperature had no sensible influence on the photochemical transformation of protochlorophyll to chlorophyll. Koski and Smith (8) showed that in dark-grown barley leaves the conversion was completely inhibited by temperatures around 90°C, and that the rate of photochemical conversion was little if at all affected by changing the temperature from 5 to 18°C (6, 9, 18).

The observations that the transformation takes place at freezing temperatures, that the rate is little affected by change of temperature in the room-temperature range, and that high temperature stops the transformation suggested that a systematic investigation of the effect of a wide range of temperatures on the transformation of protochlorophyll in situ would greatly extend our understanding of this process. Accordingly, the transformation of protochlorophyll to chlorophyll a in intact dark-grown barley leaves has been studied in the temperature range from −195 to +55°C.

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Materials and Methods

Leaf Material: Leaves from etiolated barley seedlings (*Hordeum vulgare*) were used in these experiments. The seedlings were grown in pots of sand and were watered with tap water. The temperature of the darkroom in which they were grown was about 22°C. The leaves were harvested from seedlings which had been grown from 9 to 13 days after planting. They were cut about 5 cm below the tip. During the care and handling of the plants, the only light to which they were exposed was from a flashlight screened with a dark-green cellophane filter. The exposures from the flashlight to which the leaves were subjected were ineffective for the transformation.

Analytical Methods: The percentage transformation was measured by the method employed by Koski, French, and Smith (7) in determining the action spectrum for the conversion of protochlorophyll to chlorophyll a. In brief, it consisted of grind-

ing the leaves in a mortar with sand and acetone, transferring the pigments to ether, and measuring the relative concentration of the pigments spectrophotometrically. Spectrophotometric measurements were made with a Beckman Model DU spectrophotometer at wave lengths 700, 663, and 624 mμ. At these wave lengths the specific absorption coefficients of the pigments are known (9) and from the absorption values observed the relative concentrations of the protochlorophyll and chlorophyll were calculated. The absorption of the pigments at 700 mμ is negligible so that the optical density observed at this wave length served for correcting the optical densities obtained at 663 and 624 mμ. For convenience, a curve relating the ratio of the readings at 624 and 663 mμ to the percentage transformation was used for calculating the percentage conversion. The equation used for constructing the curve was

$$T = \frac{39.9 \times 100}{(D_{624}/D_{663})\,95 + 26}$$

in which T is the percentage transformation, D_{624} and D_{663} are the optical densities observed, 39.9 and 95 are the specific absorption coefficients of protochlorophyll and chlorophyll a at 624 and 663 mμ respectively, and 26 is the difference between the specific absorption coefficients of protochlorophyll (39.9) and chlorophyll a (13.9) at 624 mμ (equation 1 (7)). In the development of this equation, it is assumed that the specific absorption coefficient of protochlorophyll is 0 at 663 mμ, and that no chlorophyll existed in the leaf previous to illumination.

From the optical density measurements, D_{624} and D_{663}, the optical density at 624 mμ due to protochlorophyll if no conversion had occurred (designated as pchl) can be calculated from the equation:

$$pchl = D_{624} + 0.275\,D_{663}$$

The constant, 0.275, is the difference of the specific absorption coefficients of protochlorophyll and chlorophyll a at 624 mμ, divided by the specific absorption coefficient of chlorophyll a at 663 mμ. This optical density can be related to the amount of protochlorophyll in the sample before undergoing any treatment and in this way the degree of loss of pigment brought about by the treatment can be estimated. In our experience, the value of D_{624} for protochlorophyll in 1 gm of untreated dark-grown barley leaves is approximately 0.1, and this value has frequently been used by us to judge the destructiveness of any treatment to which the leaves have been subjected.

Apparatus for Irradiation: Various light sources were used in the different experiments. In the experiments with polychromatic light, a 40-watt tungsten lamp was used. It was placed at the correct distance from the leaves to furnish the desired intensity of irradiation.

Monochromatic light was obtained from either a sodium or a mercury lamp. The sodium vapor lamp used was a Lab-Arc, manufactured by the General Electric Vapor Lamp Co. Light predominantly of wave length 589 mμ was obtained from the sodium lamp. To further monochromatize this light either a copper sulfate solution or a Zeiss Monochromat Filter A was placed in the light path. The intensity of the irradiation was controlled by varying the distance of the leaves from the lamp or by inserting wire screens in the light path.

The mercury lamp used was of the type Mazda 100-watt A-H4. The different spectral lines used were isolated from the general radiation by means of various filters.

The wave length 436 mμ was isolated by means of Corning glass filters No. 5113 and 3389; the wave lengths 577/579 mμ by Zeiss Monochromat Filter A; and the wave length 546 mμ by Corning glass filter no. 4303 with Zeiss Monochromat Filter B.

The intensities of irradiation used in the different experiments were measured by means of a General Electric light meter (foot candle meter, 8DW 40 Y16). This instrument was not used to measure intensities in absolute units but only in comparative units. Also it was used, comparatively, only with light from the same source and of the same quality.

The relative number of quanta per second necessary to produce a given response of the light meter at different wave lengths were:

wave length in mμ	589	579	546	436
relative no. quanta	1.046	1.000	1.060	1.196

Temperature Treatments: In the experiments carried out at temperatures from −195 to +5°C, the following procedure was used: The harvested leaves (1 gm) were placed in a vial or test tube, about 20 × 150 mm, surrounded with a metal sheath and immersed for from 30 to 60 minutes in a bath at the desired temperature. The vial or test tube was fitted with a stopper carrying a long glass tube which was open to the air but protected the leaves from the vapor of the bath. The container for the bath was an unsilvered Dewar flask of either 350 or 430 ml capacity. The lowest temperature, −195°C, was obtained by using a bath of boiling liquid nitrogen. The temperature −77°C was attained by adding solid carbon dioxide directly to an acetone bath. Higher temperatures, up to +5°C, were obtained by putting solid carbon dioxide into a long test tube which dipped into the acetone. The desired intermediate temperatures were maintained constant by manually introducing solid carbon dioxide into the test tube as required and by adjusting the carbon dioxide container to the proper depth of immersion.

After the leaves had attained temperature equilibrium with the bath in the dark, the metal shield was removed and the leaves were illuminated in the bath for the desired time. The leaf container was rotated continually, either manually or mechanically, during the exposure so that all the leaves would receive as nearly equal illumination as was possible with this set up. The light source used will be indicated in the description of the individual experiments.

In these experiments, the geometry of the apparatus was such that the exact intensity incident on the leaves could not be measured; however, the geometry

was kept constant so that in comparative experiments the intensities of irradiation should have been strictly comparable.

After the leaves had been irradiated, they were placed in the dark and flooded with 12 ml of acetone and the pigments extracted and measured in the manner already described.

In the experiments in which the leaves were treated at temperatures from +35 to +55°C, the leaves, usually 1 gm, were harvested, placed in a wire basket, and immersed in a two-liter bath of distilled water. The leaves were held in the dark at the proper temperature for the desired time, from 5 to 120 minutes, and then were quickly removed from the bath and plunged into cold distilled water. After that, they were spread out on a glass plate parallel to each other and as close together as possible and then were illuminated for 10 minutes at room temperature with light from a 40-watt tungsten lamp at 100 fc intensity. Although the transformation limits may not have been reached by this exposure, they were approached closely enough to give a true qualitative picture of the inactivating effect of temperature. The leaves were then transferred to acetone and the quantities of pigments determined.

KINETIC STUDIES: The progress of the transformation was followed in etiolated barley leaves by irradiating a series of one-gram samples with monochromatic light of constant intensity (15 fc) for various periods of time. Fixed periods of illumination were used in all experiments, namely, 0.5, 1.5, 3.0, 5.0, 7.5, 10.0, 15.0, and 25.0 minutes. The irradiations were carried out at a temperature of 6 ± 2°C in a coldroom. Since the reaction rate is little affected by temperature, this relatively crude temperature regulation was satisfactory. In order to study the conversion without interference by formation of additional protochlorophyll, a temperature considerably below room temperature was necessary.

In these experiments, the cut leaves were stored for about an hour in the dark coldroom, then laid out close together and parallel to each other on a glass plate and irradiated for the desired time at the designated intensity. After irradiation, the extent of the transformation was determined by the analytical methods already described. The wave lengths used were 589, 579/577, 546, and 436 mμ.

RESULTS

The percentage transformation of protochlorophyll to chlorophyll a at various temperatures, from −77°C to about 23°C, is shown in figure 1. The results designated with a plus sign inside a circle (⊕) were obtained with leaves which had been maintained for 60 minutes in the dark at the temperature indicated before being illuminated for 10 minutes at 100 fc intensity with light from a tungsten lamp. These results demonstrate that even at low temperatures a remarkably large photochemical transformation of protochlorophyll to chlorophyll a takes place in the leaf. However at the lowest temperature used, −195°C (not shown in the figure) there was no conversion of protochlorophyll to chlorophyll.

At −10 and −20°C, the transformation obtained by the procedure just described fell far below the curve projected from the results obtained at other temperatures. If, however, the leaves had been frozen at about −77°C and maintained at this temperature for a half hour before being raised to −10 or −20°C and equilibrated at these temperatures for 25 minutes before being illuminated, the percentage transformation was increased as the points marked with the double circle (◎) indicate. This result suggests that slow freezing damages the tissue. This is compatible with similar observations obtained with other biological material. The effect is variable, however, because in some experiments under other circumstances the conversions obtained at −10 and −20°C were consistent with those obtained over the whole temperature range.

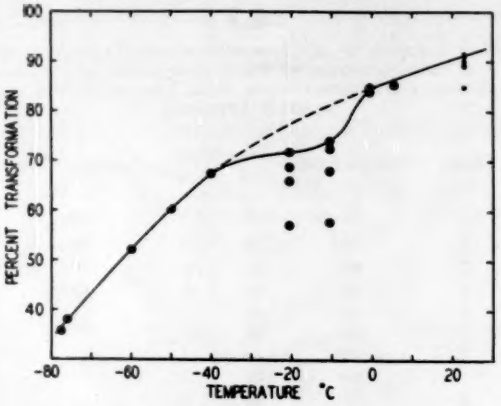

FIG. 1. The percentage transformation of protochlorophyll to chlorophyll a in etiolated barley leaves when illuminated at temperatures from −77° to 23°C for 10 min with light of 100 fc intensity supplied by a 40-watt tungsten lamp.

When the leaves were frozen at a low temperature and then thawed, they lost entirely their capacity for bringing about the protochlorophyll-chlorophyll transformation. This effect is well illustrated in the following examples: After the leaves had been frozen at the temperature of solid carbon dioxide, they were thawed in the dark and illuminated with the tungsten lamp at about 100 fc intensity. When the thawing and illumination took place in air, the transformation observed was 0%. Under these conditions, the retention of the pigment was low, about 20%. In order to avoid photooxidation, the thawing and illumination was carried out in an inert atmosphere; in carbon dioxide, 5% transformation was observed and 83% retention; in hydrogen, 0% transformation and 100% retention. These results demonstrate that even though oxidation of the pigments is prevented by using an oxygen-free atmosphere, practically no photochemical

conversion to chlorophyll occurs in leaves which have been thawed. Separate experiments demonstrated the conversion to be nearly complete when fresh etiolated leaves were illuminated in a hydrogen atmosphere.

The inhibition of the transformation at very low temperatures may be reversed at higher temperatures provided the leaves are not thawed or brought even to incipient thawing. This was demonstrated by subjecting etiolated barley leaves to various irradiation treatments at the two temperatures −69 and −40°C. The upper temperature limit was set at −40°C so as to avoid the variable effects of incipient thawing. The results of several experiments are given in table I. These results show that if there is no illumination at the lower temperature, the reversal of the temperature inhibition is complete (series no. 6 and 7). If there is illumination at the lower temperature there

TABLE I

THE REVERSAL OF THE LOW-TEMPERATURE INHIBITION OF THE TRANSFORMATION OF PROTOCHLOROPHYLL TO CHLOROPHYLL A. IRRADIATION WITH WAVE LENGTH 577/579 mμ AT 200 FC INTENSITY

SERIES	TEMPERATURE	TREATMENT DARK	TREATMENT LIGHT	TRANSFORMATION
no.	*°C*	*min.*	*min.*	*%*
1	−69	60	30	49.1
2	−69	30	30	49.2
3	−40	60	30	68.5
4	−69	60	30	61.9
	−40	30	30	
5	−69	60	15	62.8
	−40	30	15	
6	−69	60	0	66.5
	−40	30	30	
7	−69	30	0	68.2
	−40	30	30	
8	−69	60	15	52.3
	−69	30	15	

seems to be a slight permanent damage to the transformation mechanism (series no. 4 and 5).

A divided period of illumination increases the degree of transformation relatively little over a continuous period of illumination of the same duration. (Compare no. 8 with 1, 2.) This was also demonstrated in another experiment at −69°C in which two 5-minute periods of illumination were divided by 60 minutes of darkness; the percentage transformation was 34.4. With another lot of leaves, ten minutes' continuous illumination gave a transformation of 33.5 %. In both cases the intensity used was 200 fc and wave length 579/577 mμ (mercury lines).

The chief conclusion from all these experiments is that a very low temperature does not of itself permanently damage the photochemical transformation system.

The transformation obeys the reciprocity law at low temperatures. At about −76°C, illumination with 100 fc intensity (tungsten lamp) for 10 minutes yielded 38.0 % conversion while 33.3 fc intensity for 30 minutes gave 37.5 % conversion. And 100 fc intensity for 30 minutes gave 49.7 % conversion as against 49.9 % conversion brought about by 10 minutes' irradiation with 300 fc intensity. The adherence to the reciprocity law is also shown by the data in figure 2 in which the percentage transformation is plotted against the product of time and intensity. Although neither was held constant in the different runs, the points obtained fall close to a smooth curve.

The protochlorophyll-chlorophyll conversion obeys formally a second-order rate law in which the proportionality factor, k, includes the intensity of irradiation (cf equation 1). Integration of equation 1 leads to equations 2 or 3, when T is zero at zero time. By substituting T′ and t′, and T″ and t″ in equation 2, two equalities are obtained from which k can be eliminated and equation 4 obtained.

$$\frac{dT}{dt} = k(T_\infty - T)^2 = k'I(T_\infty - T)^2 \qquad (1)$$

$$k = \frac{1}{t}\left[\frac{1}{T_\infty - T} - \frac{1}{T_\infty}\right] \qquad (2)$$

$$T = \frac{kT_\infty^2 t}{1 + kT_\infty t} \qquad (3)$$

$$T_\infty = \frac{T'\dfrac{T''}{t''}(t'' - t')}{T' - \dfrac{T''}{t''}t'} \qquad (4)$$

From equation 4, the transformation at infinite time, T_∞, can be obtained from the transformations T′ and T″ observed at times t′and t″. Substitution of T_∞ in equation 2 permits k to be evaluated and placing T_∞ and k in equation 3 allows the transformation, T, to be computed for any time t.

In figures 4 and 5 the close fit of the calculated curves to the experimental points (circles) demonstrates the suitability of the second-order rate expression for calculating the progress of the reaction. The constants used for computing curves 1 and 2, figure 4, are

curve 1: T_∞, 94.1; k, 0.0190; I, 50 fc
curve 2: T_∞, 95.7; k, 0.0066; I, 17 fc

The ratio of the light intensities used was 2.94 and the ratio of the velocity constants observed was 2.88. The close agreement of these two values indicates how nearly the velocity of the transformation depends on the first power of the light intensity.

At all temperatures, the transformation follows a second-order rate law when the progress of the reaction is related to the total incident energy. This is shown in figure 2 where the percentage transformation, T (ordinate), is plotted against the product of intensity × time, i.e., foot candle-minutes (abscissa). The points represent observed values gotten at the three temperatures indicated, and the smooth curves values computed according to the second-order law. The values of T_∞ and k for the three temperatures are shown in table II. The velocity constant increases with the temperature. The energy of activation corresponding to the increase in temperature from −70.0

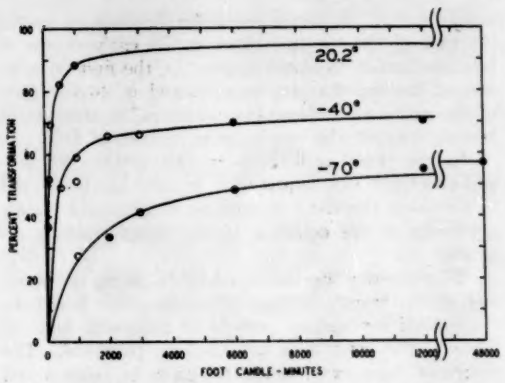

FIG. 2. The progress of the transformation of protochlorophyll to chlorophyll a in etiolated barley leaves when illuminated with light of wave length 579/577 mμ. The intensity used was either 100 or 200 fc and the time of illumination was varied from 0.5 to 240 minutes. The circles represent the values determined experimentally either at 100 fc (○) or 200 fc (●); the lines depict values calculated from the second-order rate law, equations 1 to 4, using the constants listed in table II.

to −40.0°C is about 5270 cal and from −40 to +20.2°C about 1330 cal. These values are more consistent with photochemical activation of the process than with thermochemical activation (2). The limit of the transformation, T_∞, also increases with the temperature. The data in table II show that ln T_∞ decreases linearly with increase of $1/\theta$.

Preliminary results were obtained on the effect of wave length on the transformation of protochlorophyll to chlorophyll a at low temperatures. At the wave lengths studied, 579/577, 546, and 436 mμ, the degree of transformation for a constant period of illumination increased with increase in temperature. The increase in the percentage conversion with change of temperature was practically the same for wave lengths 436 and 579/577 mμ, but was slightly, probably not significantly, larger at 546 mμ. If the positions of the absorption bands shift with temperature (5), there is no a priori reason why the ratios of the rates at two temperatures should be equal for different wave lengths.

The transformation of protochlorophyll to chlorophyll was studied also in leaves which had been heated

TABLE II

THE INFLUENCE OF TEMPERATURE ON THE VELOCITY CONSTANT AND THE LIMIT OF PHOTOTRANSFORMATION OF PROTOCHLOROPHYLL TO CHLOROPHYLL A

t	k	T_∞	$1/\theta$*	ln k	ln T_∞
+20.2	0.000132	95.3	0.00341	− 8.934	4.558
−40.0	0.0000735	71.3	0.00429	− 9.519	4.267
−70.0	0.0000138	58.4	0.00493	−11.195	4.067

* θ is the absolute temperature.

to various temperatures above room temperature. The results of these experiments are shown in figure 3. The values obtained at the same temperature are designated by the same symbol and are connected by a smooth curve.

A temperature of 35°C applied for 60 minutes before illumination had no detrimental effect on the transformation. At 39°C the effect of heating the leaves was slight, yet noticeable. At 40°C the temperature effect was definitely evident, and the damage to the transformation was about proportional to the length of time of heating. As the temperature of heating was raised, the rate of inactivation was greatly increased so that after heating for 30 minutes at 48.85°C only 6 % of the pigment was transformed by illumination. After heating at 55.3°C for 5 minutes, less than 4 % was transformed by illumination. The heat of activation for the inhibition of the transformation varies from approximately 280,000 to 60,000 cal. in going from the lower temperatures to the higher. These results suggest that denaturation of a protein is the cause for the decreased transformation (10, 14).

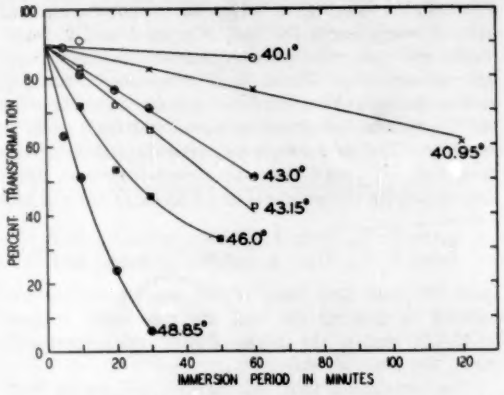

FIG. 3. The percentage transformation of protochlorophyll to chlorophyll a in etiolated barley leaves which had been immersed at the various temperatures shown in the figure for various periods of time and then illuminated for 10 minutes at room temperature with light of 100 fc intensity from a tungsten lamp.

Both the rate and the limit of the transformation are decreased by heating the leaves, as the results shown in figure 4 and in table III demonstrate.

In figure 4, the results obtained with heated (curve 3) and unheated (curve 1) leaves are compared. Both samples of leaves were irradiated at room temperature with light of wave length 589 mμ and 50 fc intensity. The heated leaves had been held at a temperature of 43.2°C for a period of 60 minutes before being illuminated. The circles designate values determined experimentally and the smooth curves values calculated according to the second-order rate law, equation 3, by use of the constants estimated from equations 2 and 4, namely,

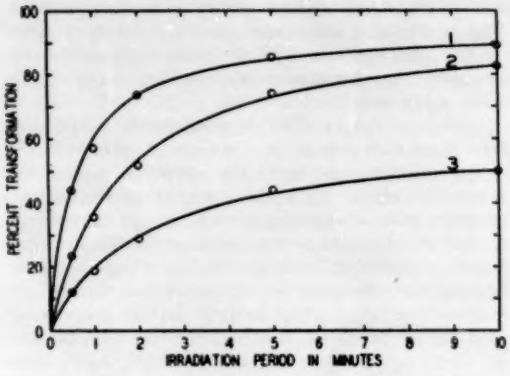

FIG. 4. The effect of light intensity and previous heating on the transformation of protochlorophyll to chlorophyll a in etiolated barley leaves illuminated with light of wave length 589 mμ. Curves 1 and 2 depict results obtained with unheated leaves illuminated with light intensities of 50 and 17 fc respectively; curve 3, results obtained with leaves heated for 60 minutes at 43.2°C previous to being illuminated with light of 50 fc intensity. The circles represent experimental data; the lines, data calculated from the second-order rate equation employing the constants listed on pages 138 and 140.

curve 1: T_∞, 94.1; k, 0.0190; intensity, 50 fc
curve 3: T_∞, 61.0; k, 0.0078; intensity, 50 fc

Both the rate and limit of the transformation are lowered by heating the leaf, the rate being reduced to 41.3 % that in the unheated leaf and, correspondingly, the limit to 64.8 %.

The conclusion that heating the leaf lowers both the rate and extent of the transformation can be drawn from further experiments in which duplicate samples of leaves were heated for definite periods of time at 43.2°C. One sample of the pair was illuminated for 0.5 minute and the other for 15 minutes with wave length 589 mμ at 50 fc intensity. The results of these experiments are given in table III. The fact that heating lowers the extent of the transformation is evidence that part of the holochrome is destroyed by heat. In this experiment, the destruction progresses nearly logarithmically with time.

TABLE III

THE EFFECT OF HEATING BARLEY LEAVES AT 43.2°C, FOR VARIOUS PERIODS OF TIME, ON THE RATE AND LIMIT OF TRANSFORMATION OF PROTOCHLOROPHYLL TO CHLOROPHYLL A. SODIUM LIGHT OF 50 FC INTENSITY WAS USED

PERIOD OF HEATING	PERCENT TRANSFORMATION OBTAINED BY ILLUMINATION FOR		LIMIT OF TRANSFORMATION CALC.	SECOND-ORDER VELOCITY CONSTANT
MIN.	0.5 MIN.	15 MIN.	T_∞	k
0	44.9	89.2	92.4	0.0205
20	20.9	71.0	77.3	0.0096
40	16.2	60.5	66.8	0.0096
80	9.7	45.9	52.7	0.0086

Heating of the leaves preceding illumination lowers the rate of the reaction more in the early stages of heating than in the later stages. In the first 20 minutes of heating the rate was reduced to 46.8 % that of the initial rate, whereas an additional 60 minutes of heating lowered the rate only an additional 5 %.

In the previous sections of this article, the second-order rate law (equation 1, p. 138) has been used to correlate the data of various experiments. How applicable is this equation to the transformation reaction?

This question was answered by following the progress of the transformation in leaves which had been illuminated for various periods of time with light of various wave lengths as described on page 137. The results of these experiments are given in figure 5 and in table IV. In figure 5 the data obtained in individual determinations are designated with dots; the average values of the percentage transformation for each period of illumination are represented by circles; and the percentages of transformation calculated from the second-order rate equation are represented by the smooth curves.

In table IV are listed the calculated and observed values of the transformation for each wave length, along with the constants used for calculation. At wave lengths 589, 579/577, and 546 mμ the calculated and observed values agree very well, but at 436 mμ there is considerable discrepancy between the two values. The cause of this discrepancy is unknown.

The excellent agreement between the observed and calculated results at the three longer wave lengths justifies the correlation of experimental data by the second-order rate equation at these wave lengths as has been done in the previous sections of this article.

DISCUSSION

The primary objective of the work reported in this paper was to analyze the mechanism of the photochemical transformation of protochlorophyll to chlorophyll a. Because the photochemical transformation of protochlorophyll occurs only when the pigment is in its natural state and not when it is dissolved in organic solvents, it is concluded that the pigment exists in the leaf in some sort of active complex, called the protochlorophyll holochrome (18).

What is the chemical nature of this holochrome? One approach for ascertaining this is to determine the effect of heating the leaf at different temperatures on the protochlorophyll-chlorophyll transformation. Heating at temperatures from 40 to 55°C lowered the limit to which the transformation proceeded. The lowering of the limit was greater the longer the time of heating, and the rate of lowering was faster the higher the temperature (fig 3). Heating the leaf in this temperature range brought about changes in the limit, and in the rate of lowering the transformation limit, similar to the effect of heating on the limits and rates of the heat denaturation of certain proteins (10, 14). Because of this, it was inferred that the protochlorophyll holochrome is a pigment-protein complex. This was also indicated by the fact that freezing and

TABLE IV

COMPARISON OF DATA OBSERVED WITH VALUES CALCULATED FROM THE SECOND-ORDER RATE EQUATION AT DIFFERENT WAVE LENGTHS. INTENSITY = 15 FC IN EVERY CASE

WAVE LENGTH	589 mμ	579/577 mμ	546 mμ	436 mμ
T_o	95.6	98.7	99.5	92.5 *
k	0.00616	0.00367	0.00264	0.00449

Time min.	*T calc.*	*T. obs.*	*T calc.*	*T obs.*	*T. calc.*	*T obs.*	*T calc.*	*T obs.*
0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.5	21.7	22.0	15.1	15.4	11.6	11.3	15.9	22.2
1.5	44.8	44.7	34.7	34.7	28.2	28.3	35.5	39.5
3.0	61.0	61.6	51.4	51.6	43.9	43.4	51.3	51.1
5.0	71.3	70.9	63.6	63.4	56.5	55.8	62.4	59.6
7.5	77.9	77.7	72.1	72.4	66.0	65.1	70.0	66.3
10.0	81.7	81.8	77.3	77.4	72.1	72.8	74.5	72.7
15.0	85.9	85.9	83.4	83.0	79.4	80.7	79.7	77.0
25.0	89.5	89.4	88.9	88.9	86.4	86.2	84.3	83.7

* An average value gotten from values increasing regularly from 88.7 to 96.3.

thawing completely destroyed the capacity for transforming protochlorophyll to chlorophyll a.

Not only did heating decrease the limit of the transformation but it also decreased the rate at which the transformation took place (fig 4; table III). The rate was lowered much more rapidly in the early stages of heating than in the later stages. In the later stages of heating, the rate was not greatly affected even though the limit of the transformation was considerably reduced. Whether heating affects an enzyme operative in the reaction, as well as the protochlorophyll holochrome, or whether it disturbs

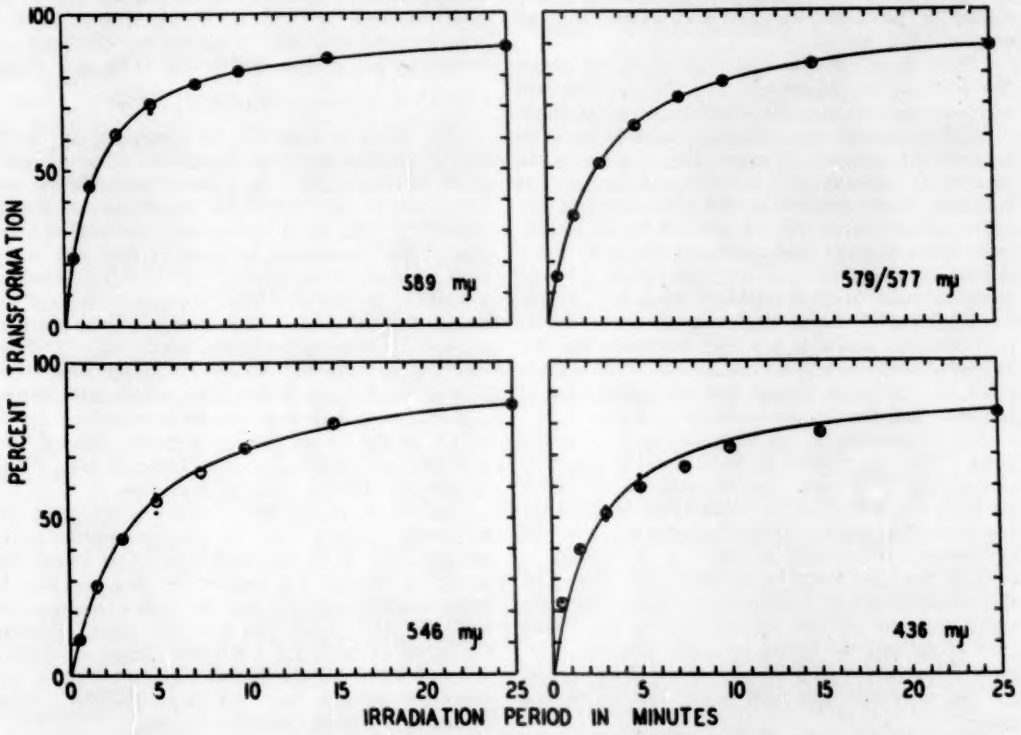

FIG. 5. The progress of the transformation of protochlorophyll to chlorophyll a in etiolated barley leaves when illuminated with various wave lengths of light (intensity 15 fc) at a temperature of 6 ± 2°C. The dots represent experimental results; the circles represent the average of the observed values; and the lines depict values calculated from the second-order rate equation by substitution of the constants shown in table IV.

the optical absorption properties of the holochrome and thus the rate, is still a matter of conjecture.

According to the commonly accepted chemistry of the conversion of protochlorophyll to chlorophyll a, the reaction is a hydrogenation (1). The question then arises concerning the mechanism of hydrogen transfer to the protochlorophyll molecule.

The fact that the reaction proceeds at low temperatures has been taken as evidence against the enzymatic nature of the reaction. This may not be strictly valid, inasmuch as enzymes are known to possess appreciable activity at low temperatures (4, 16, 17). The temperature coefficients of the enzymatic reactions are, however, of an entirely different order of magnitude from those found for the protochlorophyll transformation. For example, in comparing the activities of lipase, trypsin, and invertase at 20 and −15°C, factors of 200, 3000, and 600 times were obtained, whereas for the protochlorophyll transformation a factor of about 10 was found for the temperature interval from 20 to −70°C. Furthermore, at −70°C, no activity was observed for any of the enzymes named during a 27-day period, while the photochemical transformation of protochlorophyll proceeded quite rapidly at this temperature. Because of these facts, it is concluded that the reaction is controlled by photochemical activation rather than by enzyme activation (2, 3).

Although the reaction appears to be strictly photochemical, it does not proceed at −195°C. This lack of transformation presents something of an anomaly.

If the reaction were strictly a photochemical, intramolecular hydrogen transfer, the progress of the reaction at constant light intensity and temperature probably should conform to the first-order rate law (11). Instead of this, the reaction follows the second-order rate law under most conditions (figure 5). It is difficult to square this rate law with strictly physical processes; more likely, it relates to chemical processes. Consequently, it seems highly improbable that the photochemical action is a simple intramolecular hydrogen transfer within the holochrome. The second-order rate law is in accord with the assumption of hydrogen transfer through molecular collision.

Results obtained at low temperatures bear on this point. The transformation limit depends upon the temperature: the lower the temperature, the lower the limit (table II), and at −195°C the limit of the transformation is zero. If the conversion depends on intramolecular hydrogen transfer, it is difficult to see how the limit would be so drastically affected by the temperature. If it depends on collision between molecules, then at low temperatures in the frozen state of the leaf the sphere of action with the light-excited reactant would become more circumscribed and the transformation limit would tend to become smaller.

Inasmuch as the kinetics indicate the reaction to be bimolecular with respect to protochlorophyll, the molecular species which reacts with the protochlorophyll must occur in the same concentration as the protochlorophyll. This is probable only if the second molecular species is protochlorophyll or is in equilibrium with the amount of protochlorophyll present. This points either to the reaction between excited and unexcited protochlorophyll molecules or to the reaction between protochlorophyll and a photodissociated product of the protochlorophyll holochrome. For the reaction to accord with a second-order process, the loss of excitation energy should be rapid as compared to the intermolecular reaction.

Since the limit of the transformation is decreased when the temperature is lowered and is increased again when the temperature is raised, the effect of temperature on the limit of the transformation is reversible (table I). This is consistent with the assumption of a collision mechanism for the reaction in which the sphere of action in the frozen leaf material is reduced reversibly by lowering the temperature.

The rate of the reaction depends on the first power of the light intensity. This indicates that the bimolecular process is not dependent on the collision of two photochemically excited molecules.

From this analysis, it seems highly probable that the formation of chlorophyll from protochlorophyll is not strictly a photochemical, intramolecular transfer of hydrogen within the holochrome but requires collision processes. Whether the second reactant is another molecule of protochlorophyll or whether it is another substance that is excited by the light absorbed by protochlorophyll is still to be determined.

Summary

The effect of temperatures from −195 to +55°C on the photochemical transformation of protochlorophyll to chlorophyll a in etiolated barley leaves has been studied. At −195°C no transformation occurs. At −70°C fairly rapid and extensive conversion takes place. The conversion increases in rate and extent with increase in temperature up to 40°C. Freezing the leaves at −10 or −20°C appears to damage the transformation system. This damage can be partially avoided by freezing the leaves quickly at −77°C before raising them to −10 or −20°C for irradiation. The extent of the transformation, which is limited by a given low temperature, can be increased by illumination of the frozen leaf at a higher temperature, provided no thawing occurs. Thawing of the leaf completely prevents the transformation.

Leaves heated to 40°C begin to lose their capacity for bringing about the phototransformation of protochlorophyll to chlorophyll a. The longer the period of heating the greater becomes the loss of transformation capacity, and the higher the temperature, the more rapidly the loss takes place. Heating the leaves at 55°C for 5 minutes almost completely destroys their transformation capacity. These experiments indicate that the protochlorophyll holochrome is a pigment-protein complex.

Kinetic studies on the transformation in etiolated barley leaves at wave lengths 589, 579/577, and 546 mμ show the progress of the reaction to be rigorously a second-order process with respect to the protochlorophyll; but at 436 mμ the second-order rate law

is not strictly obeyed. The cause of this is not known. This kinetic behavior coupled with the progressive lowering of the transformation limit with lowering of the temperature suggests that the reaction is not strictly a photochemical, intramolecular process but involves intermolecular interactions. Because the reaction depends on the first power of the light intensity, it is unlikely that two photoactivated holochromes have to react.

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THE DEVELOPMENT OF CHLOROPHYLL AND OXYGEN-EVOLVING POWER IN ETIOLATED BARLEY LEAVES WHEN ILLUMINATED[1]

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When etiolated leaves are illuminated they produce chlorophyll and acquire the ability to carry on photosynthesis. By following simultaneously the development of chlorophyll and photosynthetic capacity in such leaves much may be learned concerning the participation of chlorophyll in photosynthesis. This approach has been used by several investigators, who have reached different conclusions concerning the simultaneity of chlorophyll formation and the onset of photosynthetic capacity as a brief survey of previous investigations will show.

Engelmann (4) found that all the chlorophyll-containing cells which he examined were capable of evolving oxygen when they were illuminated. Furthermore, he claimed that chlorophyll-free but etiolin-containing (protochlorophyll-containing (22)) cells of dark-grown seedlings gave off oxygen immediately upon being illuminated, which claim was verified by Ewart (5).

Irving (8) and later Willstätter and Stoll (23) examined the development of photosynthetic capacity in relation to chlorophyll formation by following the photosynthetic utilization of carbon dioxide during the greening of seedlings. Irving stated that "Etiolated leaves do not possess the power to photosynthesize CO_2 even when they have developed a large part of their chlorophyll. When the leaves have developed their full green color photosynthetic ability develops rapidly. Photosynthetic activity is not directly connected to the amount of chlorophyll produced. It must be some other component of the photosynthetic apparatus which controls the beginning of the com-

[1] Received July 2, 1953.

plete functional activity." Willstätter and Stoll, however, found that etiolated leaves, while greening, possessed great assimilatory power based on chlorophyll. With the appearance of chlorophyll the assimilatory power set in and, while the chlorophyll content of the greening etiolated leaves was small, these leaves had much higher assimilation numbers than other young leaves which had not been grown in the dark. The difference in behavior of these two types of leaves was probably caused by the fact that in the dark, chlorophyll production was inhibited by the lack of light but the formation of the enzyme factors was not.

Briggs (2) attributed the discrepancy between Irving's and Willstätter and Stoll's results to the difference in the ages of the leaves they used, because "very young leaves, no matter what their chlorophyll-content cannot function as assimilating organs . . . ."

Briggs (2) studied the pigment-photosynthesis relation in young leaves brought to different degrees of greenness. He placed such leaves in an atmosphere of hydrogen, which contained carbon dioxide, and followed their oxygen evolution during illumination. Under these conditions the leaves increased in assimilatory power without noticeable change in chlorophyll content. He concludes that an internal photochemical factor besides chlorophyll is produced which works in conjunction with chlorophyll in the photosynthetic process. This assimilatory power is built up in the dark as well as in the light because leaves which have been illuminated and placed in the dark for three days possess "an assimilatory power of the same dimensions as that of similar leaves, which have been exposed to light in the apparatus during a considerable part of three days."

Inman (7) found that etiolated leaves when illuminated began to evolve oxygen about the time the green color was visually detectable in the leaf. Some chlorophyll was formed long before oxygen evolution began.

Davis (3) demonstrated that mutant strains of *Chlorella pyrenoidosa*, although containing normal quantities of the chlorophylls, varied in their capacity to photosynthesize; the wild type absorbed carbon dioxide and evolved oxygen; one mutant did not absorb carbon dioxide but evolved oxygen; two different mutants did neither. Thus photosynthesis failed for lack of factors besides chlorophyll.

Blaauw-Jansen, Komen, and Thomas (1) found that etiolated oat leaves possessed a slight capacity for photosynthesis even when the leaves were first illuminated. This capacity increased with time and at first to a greater extent than the chlorophyll a content of the leaves. They called attention to the fact that photosynthesis increased with increase in ratio of chlorophyll b to chlorophyll a until the ratio of these pigments approached its "normal" value.

The chief questions raised by this survey are: Do etiolated leaves when first illuminated have the ability to liberate oxygen? May photosynthetic organisms contain considerable quantities of chlorophyll without possessing photosynthetic capacity? Must factors besides chlorophyll be developed before photosynthetic power is acquired? May such factors be developed in the absence of light? And, is photosynthetic capacity related to the ratio of chlorophyll b to chlorophyll a?

With the sensitive analytical methods now available for the estimation of oxygen (14) and of individual pigments (9, 10) and with the sharpening of the concepts concerning pigment formation and pigment interrelationships (11, 16, 22), it seemed possible that at least a partial clarification of the involvement of chlorophyll in photosynthesis could be gotten by a quantitative re-examination of the development of pigments and of photosynthetic capacity in etiolated leaves.

Materials and Methods

Method for Measuring Oxygen: The phosphorescence-quenching method for measuring oxygen evolution (14) was adapted to the purpose of this investigation. Preliminary reports on the modified method have already been given (17–20) and a detailed description is in preparation (21). The leaves, after having been pre-treated in the desired manner, were placed in the darkened reaction chamber of the photosynthesis apparatus. All the oxygen was removed from the apparatus by evacuation and flushing with pure hydrogen. No carbon dioxide was added to the gas stream so that whatever carbon dioxide was present came from the leaves themselves. When the phosphor had come into equilibrium with the flowing hydrogen, which required approximately 40 minutes, the leaves were illuminated for 10 minutes with light from a 25-watt tungsten lamp at 10 cm distance, and the oxygen evolved by the leaves was measured. (The light intensity on the leaf chamber was about 230 fc.)

Barley Leaf Material: In these experiments, the tips of leaves, 5 to 7 cm long, were cut from dark-grown seedlings, 9 to 11 days old. The leaves were separated into lots of known weights and were placed in glass vials with their cut ends immersed in about 1 cm of water. They were held in the dark until required for experimentation sometime during the day. The treatment given each lot of leaves is described in the appropriate place.

Pigment Analysis: All pigment analyses were performed according to the method of Koski, French, and Smith (12), which is given here only in brief outline. After the leaves had been treated as desired, they were extracted by grinding them in a mortar with acetone and sand. The pigments were transferred from acetone to ether and the pigments estimated spectrophotometrically. From the optical densities measured at 663, 644, and 624 mμ, the quantities of chlorophylls a and b and protochlorophyll were determined (9, 10, 12).

Results and Discussion

Oxygen Evolution During the Transformation of Protochlorophyll to Chlorophyll a: The first stage in the greening of etiolated leaves to be examined in relation to oxygen evolution was the transformation of protochlorophyll to chlorophyll a. For this purpose, the oxygen evolved by dark-grown

leaves during illumination was measured and compared with the protochlorophyll-chlorophyll transformation of the same leaves. The results of these experiments (summarized in table I, experiments 2 and 3) show that at most only a minute quantity of oxygen was evolved during the transformation. A control experiment (table I, experiment 1) in which one lot of leaves was carried through similar operations except for illumination, demonstrated that the method of handling produced no significant quantity of chlorophyll.

The photochemical transformation of protochlorophyll to chlorophyll a is relatively complete (~ 85 %) under these strictly anaerobic conditions. No detectable quantity of chlorophyll b is produced under these circumstances. Although the protochlorophyll-chlorophyll transformation is supposed to be a hydrogenation (6) it does not take place in the absence of light in a pure hydrogen atmosphere.

TABLE I

OXYGEN EVOLUTION DURING THE TRANSFORMATION OF PROTOCHLOROPHYLL TO CHLOROPHYLL A

	EXPERIMENT NO.		
	1	2	3
Minutes of irradiation ...	0.0	10	10
Gm of plant material	0.50	0.48	0.50
μl of O_2 * evolved	...	0.0018	0.0011
Moles of O_2 evolved	...	0.75×10^{-10}	0.44×10^{-10}
Gm of chlorophyll a formed	0.0134×10^{-6}	4.61×10^{-6}	4.11×10^{-6}
Moles of chlorophyll a formed	0.0015×10^{-6}	0.516×10^{-6}	0.460×10^{-6}
Molar ratio of O_2 evolved to chlorophyll a formed	...	1.45×10^{-4}	0.95×10^{-4}
Sum of chlorophyll a + protochlorophyll (gm)	6.09×10^{-6}	5.44×10^{-6}	5.03×10^{-6}
Percent transformation to chlorophyll a	0.2	85	82

* At room temperature (~ 25°C) and one atmosphere of pressure.

The mechanism whereby protochlorophyll is transformed to chlorophyll in the plant during irradiation is not known. From chemical considerations, the possibility exists that protochlorophyll could act as a hydrogen acceptor in the splitting of water thereby being transformed to chlorophyll a. If this happened, oxygen might be liberated according to the equation:

$$\text{Protochlorophyll} + \text{water} \xrightarrow{\text{light}} \text{chlorophyll a} + \text{oxygen} \quad \text{(Eq. 1)}$$

Inasmuch as the oxygen liberated is less than 3 % of that required by this equation, it is unlikely that the transformation follows this path.

EVOLUTION OF OXYGEN BY LEAVES EXPOSED TO CONTINUOUS ILLUMINATION: Leaves harvested from dark-grown barley seedlings were placed in glass vials containing water, about 1 cm deep, and irradiated in air for different periods of time with two 40-watt no. 4500 White Westinghouse fluorescent lamps. The leaves were about a meter distant from the lamps where the light intensity was approximately 110 fc. Two samples were irradiated at a time: one, from 0.2 to 0.3 gm, was used for subsequent measurement of oxygen evolution; the other, from 0.2 to 0.8 gm, was used for pigment analysis. The average results of three experiments are shown in figures 1A and 1B (continuous lines).

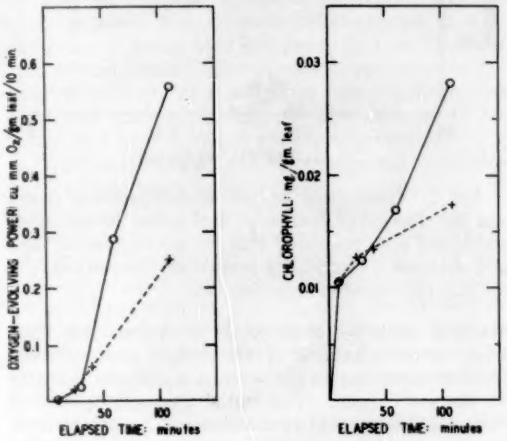

FIG. 1. Development of oxygen-evolving power (fig 1A) and of chlorophyll (fig 1B) in dark-grown barley leaves irradiated with various regimes of light and darkness. Continuous periods of illumination, ——○——; divided periods of illumination, - - - + - - -. In the experiments for the divided periods of illumination, the elapsed time is the sum of the sojourn in the dark plus 10 minutes of irradiation.

Figure 1 shows that as the period of irradiation, previous to the measurement of oxygen evolution, is increased, the development of oxygen-evolving capacity and of chlorophyll a both increase. During the initial period of exposure, the leaves acquire very little capacity to evolve oxygen although they form considerable chlorophyll. Exposures between 30 and 110 minutes bring about substantial increases in oxygen-evolving capacity, which are roughly proportional to the time (fig 1A). Chlorophyll formation is very rapid during the first minutes of irradiation; it then slows up for a short period after which it accelerates and approaches proportionality to the time of irradiation (fig 1B).

In figure 2 is shown the relationship between the chlorophyll a content of the leaves and their oxygen-

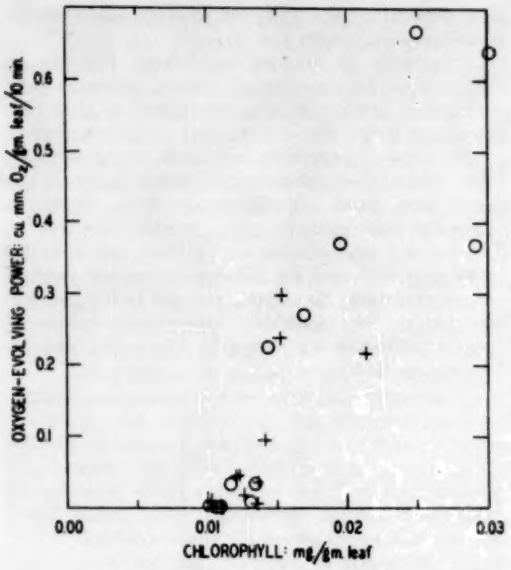

FIG. 2. Comparison of the oxygen-evolving power and the chlorophyll content of dark-grown barley leaves which had been irradiated with various regimes of light and darkness. Continuous periods of illumination, ○; divided periods of illumination, +.

evolving capacity (circles). It is obvious that relatively large quantities of chlorophyll a are formed without imparting to the leaves a significant capacity to liberate oxygen. This initial chlorophyll, formed during a short period of illumination, is derived from the transformation of the protochlorophyll originally present in the dark-grown leaves and is apparently photosynthetically inactive. As additional chlorophyll is formed, the oxygen-evolving capacity increases and, at first, about in proportion to the increase in chlorophyll.

EFFECT OF DIFFERENT REGIMES OF LIGHT AND DARKNESS ON CHLOROPHYLL CONTENT AND OXYGEN-EVOLVING CAPACITY: The effects of different regimes of light and darkness on the development of chlorophyll and oxygen-evolving capacity were also studied.

In the first regime, three pairs of leaf samples were treated in various ways and the effect on chlorophyll content and oxygen-liberating capacity determined: one pair was irradiated with the fluorescent lamps for 10 minutes; a second pair was irradiated for 10 minutes and then placed in the dark at about 20°C for 110 minutes; a third pair was irradiated for 5 minutes, then placed in the dark for 110 minutes, and again irradiated for 5 minutes. All these operations were carried out in air. After these pretreatments, chlorophyll determinations were made on one sample of the pair and oxygen-evolution measurements were made on the other. The leaf samples used for chlorophyll determinations weighed 0.5 gm and for oxygen evolution, 0.2 gm.

The results of these experiments are given in table II. They demonstrate that illumination for 10 minutes produces chlorophyll, but little, if any, capacity to liberate oxygen. Addition of a dark period to the initial irradiation increases the chlorophyll very little but increases the oxygen-evolving power very considerably. When the sojourn in the dark is followed by a second brief irradiation in air, the quantity of chlorophyll is about doubled but the oxygen liberation is increased immensely.

A considerable quantity of protochlorophyll was produced in sample no. 2 during the sojourn in the dark. This protochlorophyll was undoubtedly converted to chlorophyll during the measurement of oxygen evolution. It it is assumed that the chlorophyll so produced accounted for the oxygen evolved in this experiment then it is remarkable that a final illumination in air, which would have produced little if any additional chlorophyll, should have enlarged the oxygen-liberating power so much (sample 3).

It may be concluded that once the dark-grown leaves have been illuminated they build up an agent in the dark by metabolic processes which enables them to liberate oxygen when they are again illuminated. This agent is quickly and considerably augmented by a second brief period of illumination in air following the sojourn in the dark. It seems clear that this agent cannot be entirely identified with chlorophyll because the increase in chlorophyll is about twofold whereas the increase in oxygen-liberating capacity is about 200-fold.

In the second regime, the effect of length of dark period interposed between two 5-minute light periods was examined. The average results of three experiments are graphed in figures 1A and 1B (broken lines). The abscissas represent the elapsed time, from the beginning of the first illumination period to the end of the second, that was used preparatory to measuring oxygen evolution; the ordinates show the corre-

TABLE II

THE EFFECT OF DIFFERENT REGIMES OF LIGHT AND DARKNESS ON THE CAPACITY OF DARK-GROWN BARLEY LEAVES TO EVOLVE OXYGEN DURING A TEN-MINUTE PERIOD OF PHOTOSYNTHESIS

	SAMPLE NO.		
	1	2	3
Regime for pretreatment			
Light	10	10	5
Dark	0	110	110
Light	0	0	5
Chlorophyll			
mg per gm leaf	0.0095	0.0099	0.0177
Protochlorophyll			
mg per gm leaf	0.0012	0.0049	0.0011
Oxygen evolution			
µl per gm leaf per 10 min	0.0021	0.088	0.425
Relative chlorophyll	1.00	1.04	1.87
Relative oxygen evolution	1.0	42	202

sponding quantities of oxygen liberated per gram of leaves during ten minutes of photosynthesis (fig 1A) and of chlorophyll produced (fig 1B).

It is evident that 10 minutes of continuous illumination (zero dark period) produced, at most, only a very small capacity to liberate oxygen. As the interposed dark period was increased (fig 1A), the power for oxygen liberation was increased almost in direct proportion to the length of the dark period, discounting a short period of induction.

The amounts of chlorophyll a produced under the different irradiation regimes just described are shown in figure 1B. With 10 minutes of continuous illumination (zero dark period), considerable chlorophyll was produced. The chlorophyll was increased by interposition of dark periods, but the proportionate increase in chlorophyll was very much less than the proportionate increase in oxygen evolution.

The relationship between oxygen-evolving capacity and chlorophyll content of leaves given divided periods of illumination is shown by the crosses in figure 2. The results are similar to those obtained with continuous illumination, as comparison with the circles demonstrate.

These experimental results confirm, by a different method, what has been found by others, that chlorophyll can be present in living organisms without affording the ability for photosynthesis. Formally, increase in the ability to liberate oxygen corresponds to the increase in chlorophyll content. This relationship, however, may be fortuitous since these two properties may increase simultaneously without being functionally connected.

The great enhancement of the power to liberate oxygen induced by subjecting dark-grown leaves, which have been briefly illuminated, to a period of darkness and subsequent illumination, demonstrates that this increased oxygen-evolving capacity results from both metabolic and photochemical reactions: metabolic, because of the influence of the dark period; photochemical, because of the effect of the second irradiation.

What causes the enhancement of the photosynthetic capacity is in doubt but three possibilities exist: activation of the initial chlorophyll formed and of all the chlorophyll formed thereafter; the formation of a chlorophyll different from that derived from the original protochlorophyll; or the elaboration of substances which work in conjunction with chlorophyll.

At present nothing can be said regarding the activation of inactive chlorophyll.

The observed difference in the abilities of the chlorophyll formed from protochlorophyll and of that formed subsequently to liberate oxygen suggests that these chlorophylls are different. This interpretation is supported by the observation of Krasnovskii and Kosobutskaya (13) that the spectrum of the natural chlorophyll first formed by irradiation of etiolated bean leaves differs from that of the natural chlorophyll formed afterwards.

Some of these experiments bring evidence that substances besides chlorophyll are formed which greatly increase photosynthetic ability. The treatment, in which leaves were given a final brief irradiation following an introductory exposure to light and darkness, increases the oxygen-liberating power so tremendously in comparison with the increase of chlorophyll that the greatly increased oxygen-evolving power can hardly be reconciled with the small additional quantity of chlorophyll produced. Rather, the results point to the elaboration of substances which act in conjunction with chlorophyll.

In many instances, chlorophyll b was not present in leaves from which oxygen was evolved. From this it may be concluded that chlorophyll b is not necessary for oxygen liberation.

Summary

The development of chlorophyll and of oxygen-evolving power was followed simultaneously in etiolated barley leaves that had been illuminated under various conditions. The pigments, protochlorophyll and chlorophyll, were estimated spectrophotometrically and the oxygen evolved was determined by means of a phosphorescence-quenching method.

The transformation of protochlorophyll to chlorophyll a took place in an anaerobic atmosphere. It was not accompanied by the evolution of oxygen.

Although the etiolated leaves that had been irradiated in air for a short time possessed chlorophyll that was derived from their protochlorophyll, they lacked the power to evolve oxygen. If, after this irradiation, these leaves were stored in air in darkness, they acquired a small capacity for oxygen production. This capacity was greatly augmented by a subsequent brief irradiation.

Chlorophyll b was not necessary for oxygen evolution.

The author wishes to thank Professors James Franck, Hans Gaffron, and Dr. George L. Zimmerman, of the University of Chicago, for acquainting him with the phosphorescence-quenching method of oxygen determination and for furnishing the trypaflavine phosphor used. Also he wishes to acknowledge the helpfulness of Dr. C. S. French and Messrs. George Towner and Frank Schuster in the design and construction of the phosphorometer, and of Mr. Harold W. Milner for translating one article from the Russian, and of Mr. Allen Benitez in preparing the figures.

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MEASUREMENT OF ALGAL GROWTH UNDER CONTROLLED STEADY-STATE CONDITIONS[1]

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For reasons set forth in a following paper it has become desirable to measure growth of a unicellular alga as a function of intensity and intermittency of illumination. Conventional methods cannot be used since growth of an algal culture is accompanied by increasing mutual shading of cells. Even though the incident illumination is held constant the intensity may vary greatly between the front and back surface of the culture and the average intensity per cell decreases continually during the course of an experiment. Some special means of measuring growth must be devised.

The present paper describes an apparatus for measuring the growth rate of an alga under conditions of constant population density and constant volume in a small chamber which may be illuminated under careful control in a focused optical system. As a means of measuring growth under steady-state conditions, the method is related to the *continuous-culture apparatus* of Myers and Clark (3) and the *chemostat* of Novick and Szilard (4).

The lucite growth chamber in its final form is diagrammed in figures 1 a and 1 b. The culture cell C, 5.7 cm I. D. × 1.0 cm thick, is sandwiched between two chambers, A, for temperature control by thermostated water circulated through ports, B. Complete details of the culture cell are shown in figure 1 b. The algal suspension is stirred by a one-fourth-inch stainless steel shaft, S, driven by a small air turbine. A 4 % carbon dioxide in air gas mixture is delivered continuously into the upper portion of the cell and fresh medium is added intermittently under control of a photometric device described below. The glass overflow tube, O, serves as an automatic siphon to maintain a constant volume of suspension in the chamber. Earlier arrangements of the overflow device shown in figures 1 c and d were abandoned for reasons which will be presented later.

The complete apparatus is shown in figure 2.

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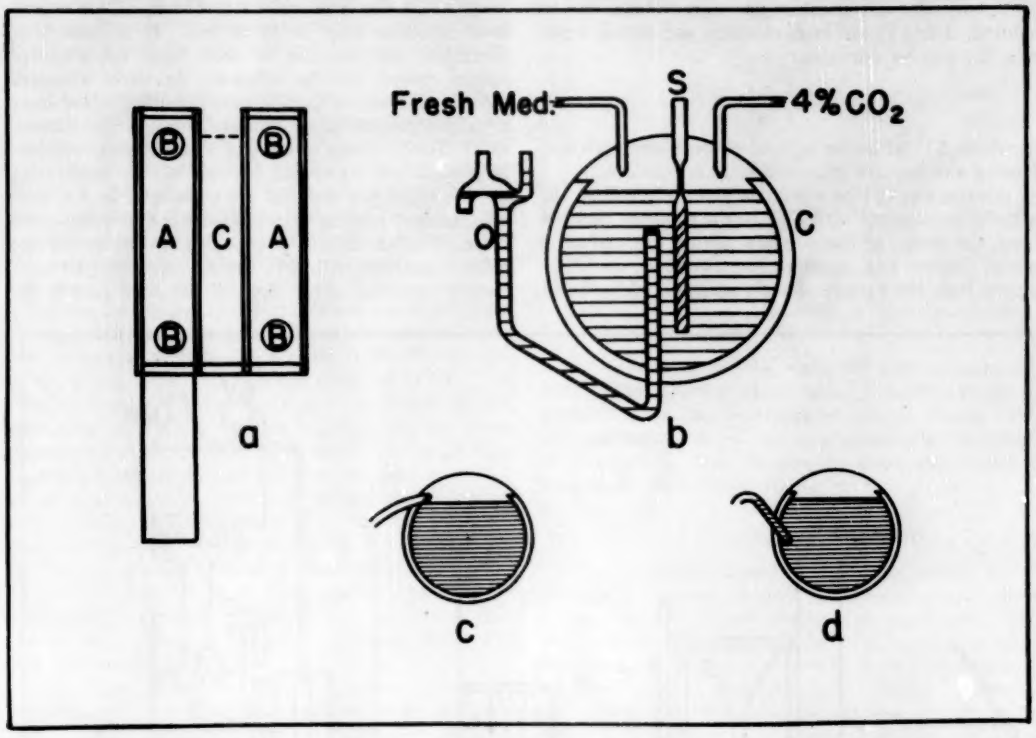

FIG. 1. Details of the growth chamber.

Light from the source, L, and spherical mirror, M, passes through a 5 cm water cell, W_1, and is focused by two 6-inch condensing lenses, CL. The uniform light field in the plane, S, is focused upon the culture cell, C, through an 18.5 cm water cell, W_2, by a Tessar-type field lens, FL, of 3 inches diameter and 7 inches focal length. Light intensity is controlled by insertion of Wratten neutral filters at F or insertion of a ground glass diffusing plate at S. Intermittency can be provided by a rotating sector placed at S. All of the apparatus behind the lens, FL, is protected from stray light so that illumination on the chamber can be cut to zero by insertion of an opaque plate, P.

The photometric control is operated by two GE barrier-type photocells: P_1, which looks through the algal suspension, and P_2, which looks directly at a portion of the final light field. A galvanometer-type relay operates from the current-balancing photocell circuit. The meter G is a Rubicon #3402 spotlight galvanometer, sensitivity 0.04 μamp/mm, with an RCA #922 phototube mounted in place of the usual glass scale. The phototube feeds into an electronic relay employing a #2050 vacuum tube and designed so that increase in light on the phototube energizes the relay. In practice, photocells P_1 and P_2 are screened so that each delivers about 100 μamp and the circuit is balanced when the transmission of the cell is about 80 %. The photometric control is similar to that previously developed by Myers and Clark (3).

The system works as follows. Multiplication in the algal suspension causes increased shading of photocell P_1. The off-balance current of the photocell circuit trips the galvanometer relay, actuating the solenoid valve, SV, and allowing fresh medium to flow into the chamber from reservoir MF until photocell balance is again obtained. Stability and sensitivity of the system are good enough to hold the density of algal suspension constant to ± 1 % for a period of several days. Concurrent with each addition of fresh medium, an equivalent small and representative sample of the algal suspension flows out of the chamber via the constant level siphon into a tared flask. The weight of suspension in the chamber (~ 20 gm) is determined at the beginning, and rechecked at the end, of each experiment. Cumulative weighings of the collected effluent are recorded periodically as a function of time.

The specific growth rate, k, is calculated by the simple relation derived as follows. The growth equation for the condition of exponential growth may be written:

$$k = \frac{1}{N}\frac{dN}{dt} \qquad (1)$$

in which dN/dt is the increment in cell number per unit time when the cell number is N. When the volume, V, in the growth chamber is held constant and when the cell number per unit volume in the chamber,

N/V, and the cell number per unit volume in the effluent, $\Delta N/\Delta V$, are held constant and equal, equation (1) can be rewritten:

$$k = \frac{1}{V}\frac{\Delta V}{\Delta t} \qquad (2)$$

in which $\Delta V/\Delta t$ is the rate of effluent accumulation. During steady-state growth $\Delta V/\Delta t$ is constant.

Attainment of the condition that $\Delta N/\Delta V$ in the effluent be identical with N/V in the chamber depends upon the design of the overflow device. In one early model (figure 1 c) in which the overflow was withdrawn from the surface of the suspension, the effluent was found to contain a higher population density than the chamber, leading to low values of $\Delta V/\Delta t$ and k. In a second model (figure 2 d), withdrawal via an inclined tube allowed return of some of the cells to the chamber during periods between dilutions, leading to a less dense effluent and high values of $\Delta V/\Delta t$ and k. The final arrangement (figure 1 b) withdraws a representative sample. Some cells do settle out in the lower part of the tube before discharge so that the collected effluent is of lowered density, but this is not important so long as the sample leaving the chamber is a representative sample.

The method purposely uses only very small quantities of cells. The chamber contains about 5 mg dry weight and the total collected effluent of an experiment contains only 10 to 20 mg. It is important, therefore, that no loss of cells from the chamber occurs except via the effluent. In early attempts stirring and carbon dioxide were provided by bubbling a carbon dioxide-in-air mixture through the suspension. The breaking of bubbles at the surface resulted in a small but significant loss of cells by splattering on the upper dry walls of the chamber. In the final arrangement stirring is provided by a stainless steel rotor. Carbon dioxide is provided by saturating the influent medium with 4 % carbon dioxide in air; the medium contains about half of the total carbon requirement of the cells produced, and the remainder is furnished by diffusion into the suspension from the 4 % carbon dioxide in the gas phase maintained at the surface. With this arrangement no carbon dioxide limitation of growth was expected, nor observed even at very high light intensities.

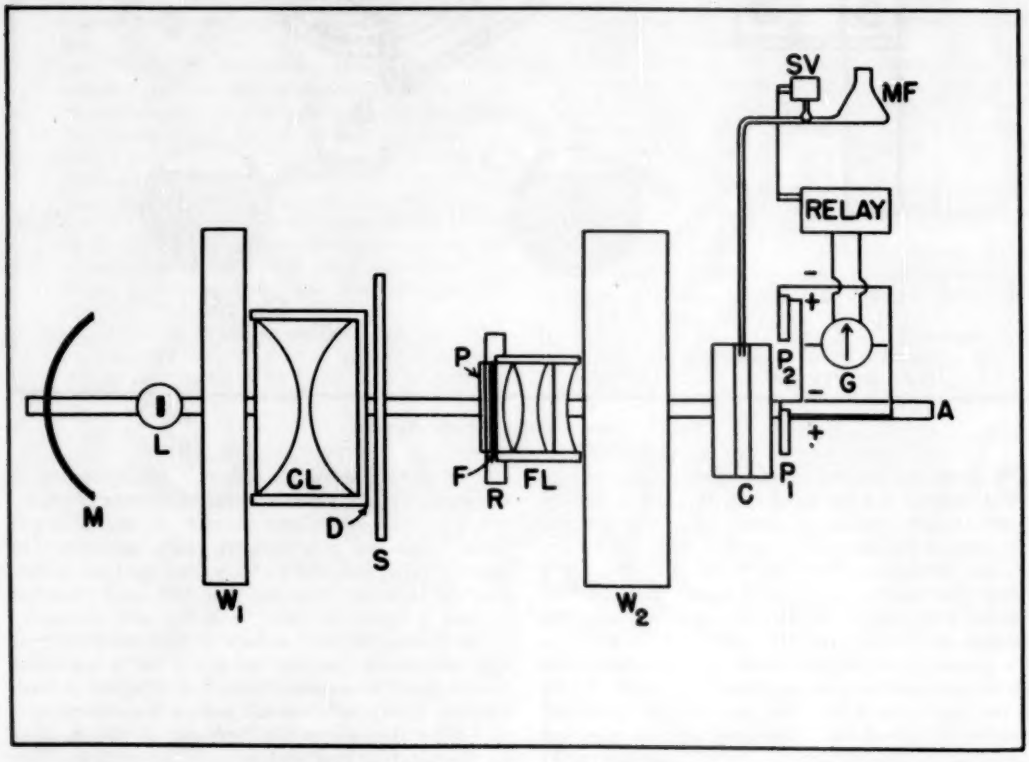

FIG. 2. Details of the complete apparatus.

In initial trials the light source was a 1000-watt, 120-volt projection lamp operated at 100 volts from a voltage stabilizer and variable transformer. Light intensity was measured by a calibrated large surface Moll thermopile or a calibrated photronic cell inserted in place of the growth chamber. Variation in intensity due to lamp decay could be held to $\pm$ 2 % of the mean value over a three-day period. Light intensity

over the surface of the growth chamber, checked by scanning the beam with a small vacuum thermocouple having a 2 mm diameter receiver, proved uniform to ± 2 %.

The apparatus can be applied to growth rate studies of any unicellular alga which suspends uniformly in liquid culture. To date it has been applied only to the Emerson strain of *Chlorella pyrenoidosa* at 25°C. Additional details of operation and typical experimental results may be cited for this alga.

The culture medium was a modified Knops solution now used routinely in this laboratory. It contained 0.010 M $MgSO_4$, 0.012 M KNO_3, 0.009 M KH_2PO_4, 0.5 gm per l of the chelating agent, ethylene diamine tetraacetic acid, and trace elements in parts per million as follows: 30 Ca, 20 B, 10 Fe, 20 Zn, 4 Mn, 4 Mo, 1 Co. The pH was adjusted to 6.8. The solution was used full strength in a continuous culture apparatus (3) which provided a uniform and pure culture inoculum for each experiment. In the growth-measuring apparatus the medium was used at a 1 : 3 dilution to minimize formation of slight precipitates. It has been demonstrated that growth rate is independent of total concentration of Knops solution within this dilution range (2). No attempt was made to maintain pure culture technique in the growth-measuring apparatus. Under the conditions employed, contamination by other microorganisms was not significant.

The data of five successive experiments run at different light intensities are presented in figure 3 and table I. The linear character of the curves of figure 3 provides an over-all measure of the stability and sensitivity of the system in maintaining steady-state conditions. Precision of the slope of each curve was estimated by the Student t distribution using a 95 % confidence limit; in order to use the Student t distribution, a normal distribution of errors in slope from the true slope was assumed. In practice, the values of k determined graphically are regarded as accurate to ± 0.05 day^{-1}. Treatment of growth rate as a function of light intensity is reserved for the following paper.

TABLE I

REPRESENTATIVE DATA ON GROWTH OF *Chlorella pyrenoidosa* AT 25°C UNDER DIFFERENT LIGHT INTENSITIES

EXPERIMENT *	V	ΔV/Δt	k **
no.	*ml*	*ml/day*	*day^{-1}*
54 a	22.1	42.3	1.92 ± 0.05
55 b	21.7	32.0	1.48 ± 0.09
57 c	22.0	23.5	1.07 ± 0.05
58 d	22.0	15.0	0.68 ± 0.07
56 e	21.9	8.16	0.37 ± 0.01

* Letters designate corresponding curves of figure 3.
** The dimension of specific growth rate of day^{-1} is that required by equation (1) and is equivalent to a dimension of $\log_e$ units/day for the integrated form of equation (1). The ± variation indicated is that calculated for a 95 % confidence limit of the regression coefficient for ΔV/Δt.

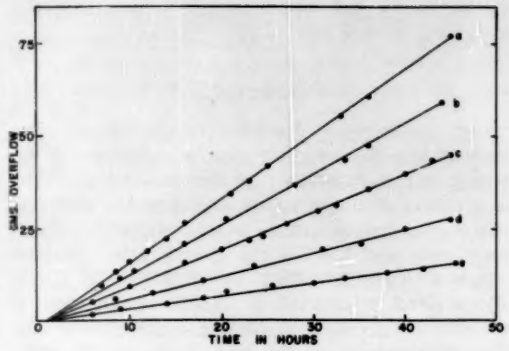

FIG. 3. Plots of the data of five experiments. Accumulated overflow versus time. Measured weight in grams is taken as equivalent to volume in ml. The slopes of the curves give the corresponding ΔV/Δt values cited in table I. Data for curves d and e were extended beyond the time period shown.

DISCUSSION

The apparatus described is one of several which provide a constant density of population and allow study of growth under steady-state conditions. The *chemostat* of Novick and Szilard (4), applied in principle also by Monod (1), may be described as a continuous-dilution, constant-volume device. Rate of growth (which always must be less than the maximum rate) is established by a constant dilution or washing-out rate, $\frac{1}{V} \times \frac{\Delta V}{\Delta t}$. The population density then adjusts itself to the maximum value permitted by some limiting component of the influent medium. The *chemostat* is particularly adapted to the study of growth and mutations of microorganisms under deficiency conditions.

The *continuous-culture apparatus* of Myers and Clark (3) may be described as a controlled-dilution, variable-volume device. Dilution is controlled by a photometric device to just such a rate as will keep the density constant and balance the rate of growth. Volume is allowed to increase and effluent samples are withdrawn periodically to some fixed volume. The specific growth rate, k, can be evaluated from the integrated form of the growth equation as $\frac{1}{t - t_o} \ln \frac{V}{V_o}$, where V and V_o designate the volumes corresponding to times t and t_o. The method can be used to measure rate of growth and to provide uniform experimental material.

The apparatus herein described is a controlled-dilution, constant-volume device. Dilution is controlled by a photometric method to maintain constant density. Maintenance of constant volume by means of an overflow allows direct use of the differential form of the growth equation.

A continuous-dilution, variable-volume device utilizing the principle of Novick and Szilard is also possi-

ble in theory but would require a logarithmically increasing dilution rate.

Summary

An apparatus is described for precise measurement of growth rate of an alga as a function of intensity and intermittency of illumination. Dilution of a culture is controlled by a photometric device to just such a rate as will maintain constant density of population and balance the growth rate. Maintenance of constant volume by an automatic siphon allows direct application of the differential form of the growth equation in evaluating specific growth rate. The relationships of the new apparatus to other steady-state growth devices are discussed.

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GROWTH RATE OF CHLORELLA IN FLASHING LIGHT[1]

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Studies on the mass culture of algae under sunlight conditions have demonstrated that the most important factor limiting the yield per unit area of exposed surface lies in the characteristic of light saturation (12). Cells at the front surface of a culture use the very high light intensity of sunlight with low efficiency; at the same time cells at the back surface of a dense culture may receive no light at all. In theoretical studies of effects of intermittent light on photosynthesis it has been shown that light of high intensity may be used with high efficiency if presented in short flashes separated by long dark periods. One anticipates that some similar effect will hold for the total growth process as well as for photosynthesis alone. If, by turbulence of culture suspension, individual algal cells are moved back and forth between the high intensity of the front surface and the darkness of the back surface, an improvement in over-all efficiency of light utilization by the culture might be effected.

Reported herein is an investigation of the characteristics of growth of a representative alga, *Chlorella pyrenoidosa*, in intermittent light. In order to obtain interpretable results, rate of growth has been studied in thin layers of culture suspension of such low population density that mutual shading effects of the cells are minimized. Intermittent illumination of measurable characteristics has been provided by a mechanical optical system. As a secondary objective it has been necessary to determine also the characteristics of the light intensity curve for growth in continuous illumination.

Materials and Methods

The growth rate at each light intensity or intermittency regimen was determined by a separate experiment. Cells harvested from a continuous-culture apparatus were centrifuged out and resuspended in a ¼-strength EDTA Knops solution (13) to a population density which gave a 50 % transmission in an Evelyn colorimeter with 600 mμ glter. In the growth chamber this suspension had a transmission of 81 % as measured with a fully-illuminated photocell placed behind the suspension. Constancy of the photometric control was checked during and at the end of each experiment by readings of transmission of the suspension in the Evelyn colorimeter; all such readings fell within the range of 48–52 %. In each experiment the specific growth rate, k, was evaluated in $\log_e$ units per day as described in the preceding paper. The temperature used throughout was 25°C.

Intensity Measurement: Measurements of light intensity at the position of the growth chamber were made with a large surface Moll thermopile with a compensated Aryton shunt and Rubicon Type T galvanometer. It was calibrated without a shielding window against an NBS standard lamp. For subsequent measurements the thermopile was used with a thin glass shielding window. Estimate of the intensity of visible radiation was obtained by use of a Jena RG8 filter which transmits the near infrared and has a sharp cut-off at 7000 Å (fig 1). Readings with the thermopile were taken alternately with the system open, with the RG8 filter, and with the system closed by a shutter. (For positions of the shutter and filter in the optical system see figure 2 of the preceding paper.) Total deflection with the system open, minus deflection with the RG8 filter was taken as the measure of visible radiation. The procedure is similar to that used by Kok (8). A series of check readings made with a calibrated Weston 603 Illumination meter showed good linearity with the thermopile and RG8

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filter method at lower light intensities. In the intensity data to be presented below, 41 erg/cm²-sec is equivalent to 1.0 foot candle.

LIGHT INTENSITY CONTROL: The light source was a 1000-watt, 120-volt projection lamp operated at 100 volts ± 0.5 volt from a Raytheon voltage stabilizer and variable transformer. In the experiments with intermittent light the voltage was increased to 105 volts in order to obtain a somewhat higher intensity. This makes a small but not important change in the spectral quality of the light used in the two series of experiments. The intensity for each experiment was taken as the average of values obtained at the beginning and end which, except in two very long experiments, were within 2 % of the average.

For study of growth rate as a function of intensity of continuous illumination the intensity was varied by use of Wratten neutral filters or copper screens in position F of the optical system. Lower intensities required addition of a ground glass diffusion plate in position S. Transmission versus wave length curves of the neutral filters, the RG8 filter, and the thermopile window, as determined with a Beckman spectrophotometer, are presented in figure 1. The neutral filters are reasonably flat in the 5000 to 7000 Å region which contains 86 % of the visible radiation used (fig 2).

SPECTRAL CHARACTER OF THE ILLUMINATION: The spectral distribution of the illumination used is presented in figure 2. Curve A is the relative energy output of the lamp operated at 100 volts, color temperature 2990°K, with corrections for tungsten emissivity (data kindly supplied by Dr. H. Haynes of General Electric). Curve B is the relative energy output of the lamp transmitted by 23.5 cm of water as calculated from the data of Collins (3). Curve B represents the spectral distribution of the illumination seen by the algal suspension. Curve C is the fraction of the total illumination (curve B) transmitted by the RG8 filter.

The method of estimating intensity in the visible region described above, effectively subtracts the area under curve C (transmitted by the RG8) from the area under curve B (total illumination). Actually

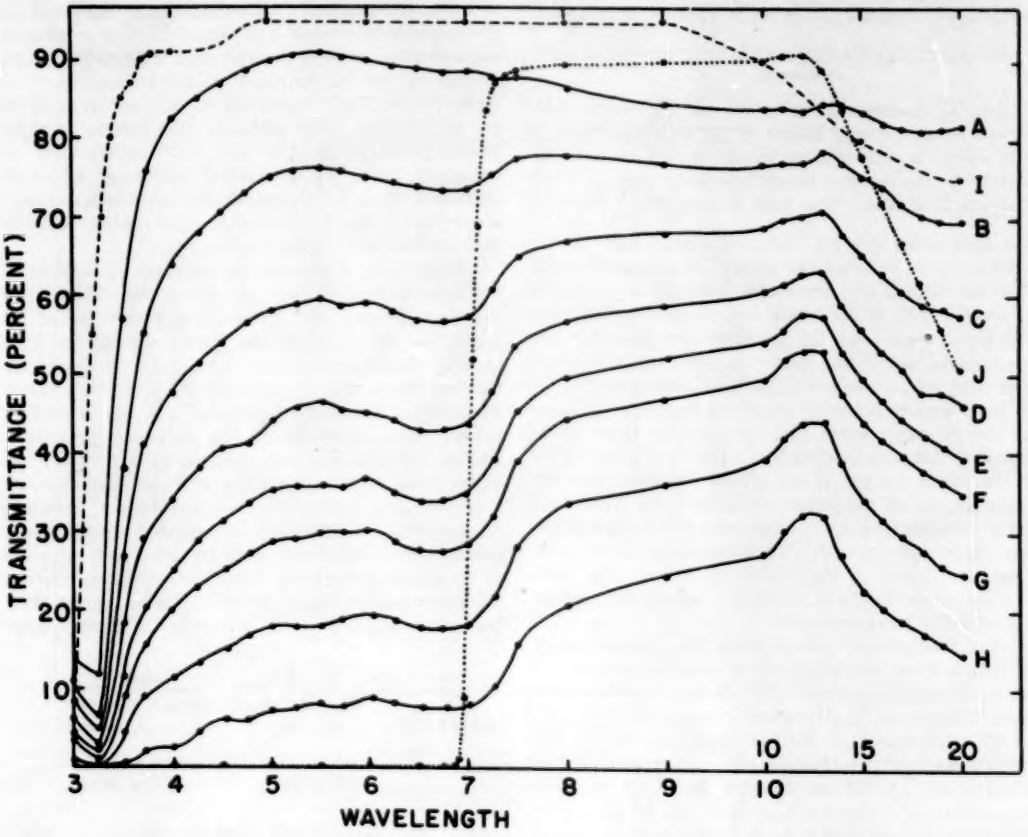

FIG. 1. Transmission curves of the filters used. A–H, the series of Wratten neutral filters. I, the thermopile window. J, the RG8 filter. The unit of wave length is 10^3 Å.

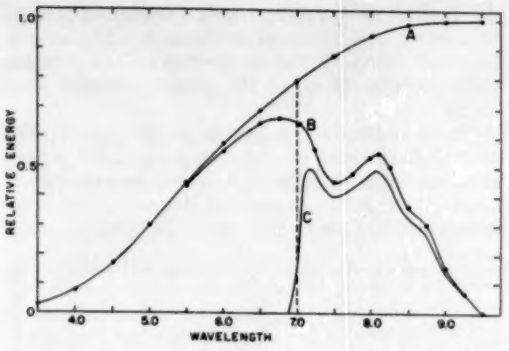

FIG. 2. Spectral characteristics of the illumination. A, the relative energy output of the 1000-watt lamp at 100 volts. B, the relative energy passed by 23.5 cm water. C, the relative energy passed by 23.5 cm water plus the RG8 filter. The unit of wave length is 10^3 A.

the area under curve C includes a small part of the visible region and excludes about 7 % of the infrared. The net effect is that the visible intensity as estimated is about 105 % of the true value. This error is almost exactly balanced by the window correction for the thermopile which was calibrated without a window but used with a window of 95 % transmission (fig 1). A more serious intensity error lies in reflection losses at the air-lucite-water and water-lucite-water interfaces of the growth chamber. The net transmission of the front surface of the growth chamber into the algal suspension was estimated at 85 % by differential measurements on a lucite absorption cell using a Beckman spectrophotometer. In the intensity data to be presented, none of the above corrections has been applied since they are entirely constant throughout the series of measurements.

INTERMITTENCY CHARACTERISTICS: Intermittency of illumination was obtained by rotating sectors. A series of aluminum disks 1/16″ thick and 40″ circumference were cut to give sector openings of 1/4″, 1″, or 4″ as measured at the periphery. A disk in use was mounted on the shaft of a gear ratio box coupled directly to a 1/5 HP synchronous motor. A complete description of important characteristics of the sector disks used is presented in table I.

The position of a sector disk in the optical system (fig 1, S, of the preceding paper) is such that it cuts the uniform emerging beam from the condensing lens, and its image is sharply focused upon the center plane of the growth chamber. As a sector opening crosses the light beam its transmitted bar of light sweeps across the growth chamber.

For simplicity of interpretation of the end results it is desirable that the light flashes seen by each algal cell are sharply defined so that a plot of intensity against time takes a square-wave form. Some small and unmeasurable error is introduced since the algal cells are themselves moving erratically in the stirred suspension. For any one sector and rpm the lengths of a light flash as seen by different cells probably falls on a distribution curve with mean value at the calculated length (table I). A second type of error, due to lens aberrations and the scattering by the suspension itself, causes a smearing of the square-wave form. The time characteristics of a light flash have been estimated by a simple method. With the disk at rest and an edge of a sector opening in a vertical position, a photocell with a vertical 0.25 mm diaphragm was moved horizontally across the edge of the light field in the plane normally occupied by the growth chamber. Galvanometer deflections were recorded as a function of horizontal position of the diaphragm and the data are presented as curve A of figure 3. The procedure was repeated with a typical algal suspension in an absorption cell 0.5 cm thick (curve B) and again in a cell 1.0 cm thick (curve C) placed just in front of the diaphragm. The intensity distribution in light flashes produced by 1/4″ and 1/2″ sectors may be visualized from the completed curves of figure 3. Curves A, B, and C represent the light flash contours occurring at the front surface, the center, and the back surface of the algal suspension.

While figure 3 presents an adequate description of the light flashes, it does not completely characterize the dark periods. Of critical importance is the integrity of the darkness maintained during the dark periods, particularly when these are very long, for reasons which will be considered in the subsequent discussion. The characteristics of a typical long dark period were examined by the following procedure. The growth chamber was replaced by a photocell receiver faced with a diaphragm of 3 mm diameter and a ground glass plate. A sector with four 1″ openings (Experiment 73, table III) was rotated manually and galvanometer deflections were recorded as a function of peripheral movement. The larger diaphragm opening gives poorer resolution of the edge of the flash but allows more accurate estimation of light leakage

TABLE I

SECTOR CHARACTERISTICS

EXPT.	SECTOR SIZE	OPENINGS	DISK SPEED	t_f	t_d	$\frac{t_f}{t_f + t_d}$	FREQ.
no.	*in.*		*rpm*	*sec*	*sec*		*sec*$^{-1}$
82	4	1	90	0.0667	0.600	0.100	1.5
81	4	2	90	0.0667	0.266	0.200	3
80	4	4	90	0.0667	0.100	0.400	6
75	1	2	90	0.0167	0.316	0.050	3
73	1	4	90	0.0167	0.150	0.100	6
74	1	8	90	0.0167	0.067	0.200	12
77	1	12	90	0.0167	0.039	0.300	18
66	1	1	360	0.00417	0.163	0.025	6
67	1	2	360	0.00417	0.079	0.050	12
65	1	4	360	0.00417	0.037	0.100	24
68	1	8	360	0.00417	0.017	0.200	48
78	1	12	360	0.00417	0.010	0.300	72
70, 72	1/4	4	360	0.00104	0.041	0.025	24
69	1/4	8	360	0.00104	0.020	0.050	48
71	1/4	16	360	0.00104	0.009	0.100	96
79	1/4	24	360	0.00104	0.006	0.150	144

during the dark period. The data are presented in figure 4.

CHLOROPHYLL CONCENTRATION OF THE CELLS: At the termination of each growth experiment two supplementary determinations were made on the final culture suspension. Relative chlorophyll concentration was determined by a Beckman spectrophotometer on a 10 ml solution obtained by extracting the cells from a 5.0 ml aliquot of suspension in boiling methanol. Cell volume was determined by centrifuging aliquots of suspension to constant packed volume in calibrated capillary tubes.

with increasing intensity. An approximate value of intensity for light saturation may be estimated at 2.5×10^4 erg/cm^2-sec where the growth rate attains about 90 % of its observed maximum. This value, corresponding to about 600 fc, is considerably higher than the 100 fc which can be obtained from a similar curve of Myers (11). The present value of 600 fc was obtained under unilateral illumination; the earlier value of 100 fc was obtained in an annular chamber illuminated by four tubular lamps and the reflections from a surrounding housing. The discrepancy illustrates an error easily made in the design and interpretation of experiments concerned with evaluation of light intensity effects. Measurements of critical values of intensity made under multi-directional illumination do not provide data which submit to general interpretation. Error of interpretation arising from these considerations was made by Myers (11) and has been made by others in interpretation of the results of Hoover, Johnston, and Brackett (7) on wheat leaves obtained under similar conditions of multi-directional illumination.

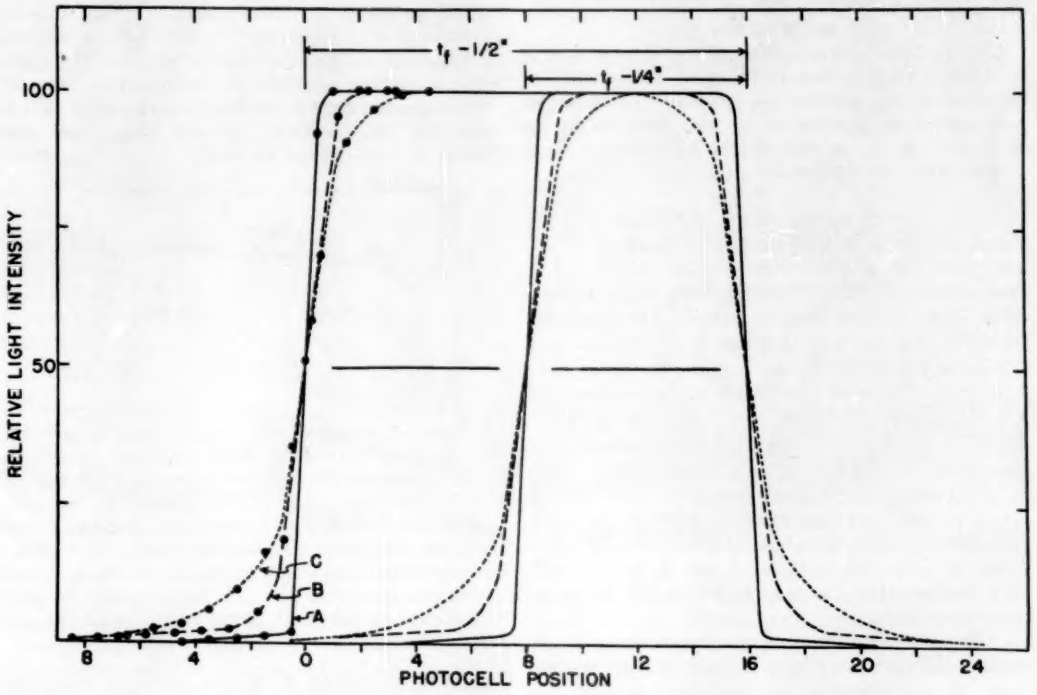

FIG. 3. Time characteristics of the light flash as seen by algal cells at the front surface (A), the center (B), and the back surface (C), of the growth chamber. See text.

RESULTS AND DISCUSSION

CONTINUOUS ILLUMINATION: Experiments measuring the specific growth rate as a function of intensity of continuous illumination are summarized in table II. The data are plotted in figure 5 as solid points connected by a solid line to form the expected saturation curve. There is a small anomaly in the points at very low light intensity which appear to cause a break in the curve. However, since small values of the growth rate are established with lowered precision, no significance is attached to the apparent anomaly. The extrapolated value of the compensation point for growth can be estimated only as a value less than 1000 erg/cm^2-sec or 24 fc.

At high light intensities, growth rate is not completely light-saturated but continues to increase slowly

INTERMITTENT ILLUMINATION: The series of experiments on growth rate as a function of intermittency are summarized by table III. Characteristics of intermittency in each experiment are defined by the flash time, t_f, the dark time, t_d, and the fractional time in the light, $t_f/(t_f + t_d)$, the initial light intensity, I_o, and the integrated light intensity, I_a, on the growth

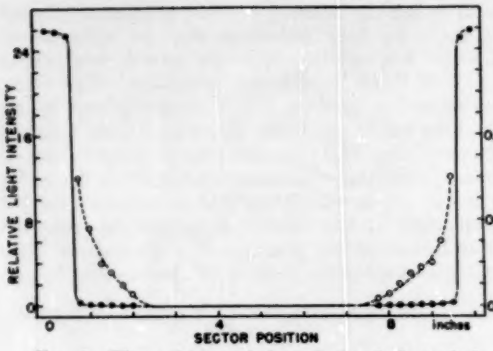

FIG. 4. Time characteristics of the dark period as determined using a disk with four 1" sector openings (experiment 73). Relative light intensity (galvanometer readings) versus position of the disk measured at the periphery. ●——● original data, left scale; ○---○ original data × 40, right scale.

chamber. It was desired that I_o (measured with the sector removed) be kept constant throughout all experiments but in practice this value varied ± 7 % from a mean of 228×10^3 erg/cm²-sec, owing to lamp decay and variation between lamps. The integrated intensity, I_a, was calculated as $I_o \times t_f/(t_f + t_d)$ and measured directly by the thermopile with the sector in operation. Values of I_a obtained by the two methods are not in very good agreement. The disk of experiment 79, which showed the most severe discrepancy, was carefully checked with a cathetometer. Individual openings showed variations of ± 12 % in width and area and a total open area of 0.749 of the nominal open area ($t_f/(t_f + t_d)$ of table I). The I_a (measured) of table III is 0.751 of the I_a (calculated). Only the measured values of I_a are used in the subsequent treatment.

Effects of intermittent light on photosynthesis and growth can be treated as a problem in light integration. If photochemical intermediates produced in a short flash of high intensity can be processed further by enzymatic reactions during the following dark period, then the cell is accomplishing a time integration of the light energy received. Following this line of argument, as proposed by Kok (8), growth rates in intermittent light have been plotted in figure 5 against integrated intensity (I_a, measured, table III). The resulting curves A, B, C, D have parameters of flash time t_f. A base line, (X), may be drawn for the limiting case in which reactions leading to growth occur at maximum rate during the light flashes but not at all during the intervening dark periods, ie, for zero integration. Calculation of values on curve X are made simply as $2.1 \times t_f/(t_f + t_d)$ where 2.1 is taken as the maximum growth rate in continuous light.

Comparison of the curves of figure 5 gives a measure of the degree to which the alga integrates intensity when light of high intensity ($\sim 230 \times 10^3$ erg/cm²-sec) is presented in flashes of various lengths. If integration is perfect, a curve for intermittent light will superimpose the curve for continuous light. It will be seen that this condition is closely approached when flashes of 0.001 second are used. As the flash time is

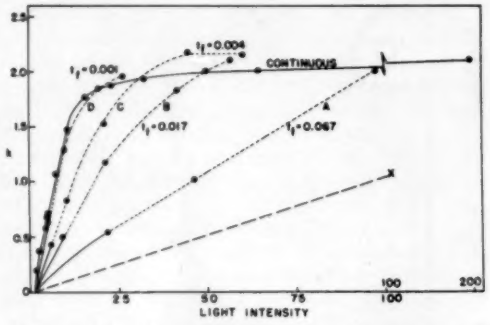

FIG. 5. Growth rate versus light intensity. Solid line: in continuous illumination (table II). Broken lines: in intermittent light (table III) for the flash time noted on each curve and with the integrated intensity determined by the length of the dark period. The unit of light intensity is the kiloerg/cm²-sec.

increased (curves C, B, A) the degree of integration becomes lower and the curves approach the base line, X, of zero integration. An unexpected character of curves B, C, and D is their approach toward higher growth rates than those observed at equal integrated intensities of continuous light.

A second and more conventional treatment of flashing light data is based on yield per flash as a function of dark time, following the argument of Emerson and Arnold (5). In measurements of photosynthesis yield per flash may be expressed in $\mu l\ O_2/\mu l$ cells per flash. The specific growth rate k (as will be demonstrated below) has dimensions equivalent to $\mu l\ O_2/\mu l$ cells per unit time. Growth yield per flash may be expressed in terms of k/flash, as in the ninth column of table III. The data are plotted in figure 6 to give a family of curves of flash yield against dark time for the four values of t_f used. As t_d approaches zero, the yield per flash approaches the value of k/t_f observed in continuous illumination at high light intensity. This limit yield per flash at zero t_d was negligible for the very short flashes (10^{-5} sec) used by Emerson and Arnold. In the present experiments,

TABLE II

GROWTH RATE AS A FUNCTION OF INTENSITY OF CONTINUOUS ILLUMINATION

EXPERIMENT	INTENSITY	k
no.	*erg/cm²-sec × 10³*	*log. units/day*
60	1.54	0.20
56	2.12	0.37
61	4.10	0.58
58	4.55	0.68
57	7.63	1.07
55	11.2	1.48
59	16.0	1.77
62	20.3	1.88
54	26.4	1.92
76	49.7	2.00
64	65.3	2.01
63	196.0	2.10

TABLE III

GROWTH IN INTERMITTENT ILLUMINATION

EXPT.	t_f	t_d	I_o	I_a (CALC.) *	I_a (MEAS.)	k	GAIN-k **	k †	GAIN-k
no.	*sec* $\times 10^{-3}$	*sec* $\times 10^{-3}$	*erg/cm²-sec* $\times 10^3$	*erg/cm²-sec* $\times 10^3$	*erg/cm²-sec* $\times 10^3$	*day*$^{-1}$	*day*$^{-1}$	*flash*$^{-1} \times 10^{-7}$	*flash*$^{-1} \times 10^{-7}$
82	66.7	600	218.0	21.8	21.5	0.54	0.33	41.7	25.5
81	66.7	266	226.5	45.3	46.5	1.20	0.78	46.3	30.1
80	66.7	100	241.2	96.5	97.3	2.01	1.17	38.8	22.6
75	16.7	316	211.1	10.6	9.88	0.50	0.40	19.3	15.4
73	16.7	150	221.1	22.1	21.2	1.18	0.97	22.8	18.7
74	16.7	67	222.5	44.5	42.1	1.83	1.41	17.7	13.6
77	16.7	39	212.2	63.7	57.4	2.11	1.48	13.6	9.52
66	4.2	163	237.6	5.94	5.75	0.42	0.37	8.10	7.14
67	4.2	79	239.5	12.0	10.4	0.83	0.72	8.01	6.94
65	4.2	37	228.9	22.9	21.7	1.53	1.32	7.38	6.37
68	4.2	17	239.6	47.9	44.5	2.18	1.76	5.26	4.24
78	4.2	10	225.6	67.7	60.4	2.15	1.52	3.46	2.44
70	1.0	41	222.3	5.56	4.83	0.63	0.58	3.04	2.80
72	1.0	41	231.8	5.80	4.97	0.71	0.66	3.42	3.18
69	1.0	20	223.5	11.2	9.54	1.29	1.18	3.11	2.85
71	1.0	9	230.8	23.1	19.4	1.84	1.63	2.22	1.97
79	1.0	6	241.2	36.2	27.2	1.96	1.64	1.58	1.32

* $I_o \times \frac{t_f}{t_f + t_d}$.

** $k - \frac{t_f}{t_f + t_d} \times 2.10$.

† $\frac{k}{\text{flashes/sec} \times 8.64 \times 10^4 \text{ sec/day}}$.

however, the limit yield at zero t_d is not negligible for the longer light flashes. The curves of figure 6 describe not only the effects of intermittency but also the effects of continuous illumination operating during the significantly long periods of the light flashes. The latter effects may be eliminated by subtracting the value of k on curve X of figure 5 from the observed value of k in flashing light of the same integrated intensity. This procedure has been applied to give the data, labelled "gain-k" in the eighth column and "gain-k/flash" in the tenth column of table III, which characterize only the intermittency effects. Actually the procedure makes some degree of overcorrection; it is based on the assumption that the photochemical apparatus is maintained at a constant degree of light saturation throughout the length of the light flash and independent of the length of the preceding dark period.

Yield per flash in terms of gain-k is plotted in figure 7 as a function of t_d. With increasing dark times the flash yields approach maximum values. (No explanation is apparent for the somewhat decreased flash yields observed for the longer flash times and very long dark periods and no particular significance is attached to this aspect of the data.) The maximum flash yield (and also the dark time required to obtain half-maximum flash yield) show a saturation curve when plotted against t_f, as in the insert of figure 7.

Comparison of the present data on specific growth rate with those of photosynthesis studies requires conversion of k to the units measured by manometry. When k = 1.0 $\log_e$ unit per day, 1.0 μl cells will produce 1.0 μl of new cells per day. 1.0 μl of cells, determined as the minimum packed volume on centrifugation, is equivalent to 0.24 mg dry weight and 120 μgm or 10 μmols of carbon. The equivalents are based upon previously published values from this laboratory and show only minor variation with light intensity. Ten μmols of carbon require an uptake of 224 μl carbon dioxide. Conversion to oxygen evolved is attended by some uncertainty as to the proper choice of assimilatory quotient. At low light intensities and with nitrate as the nitrogen source, the quotient

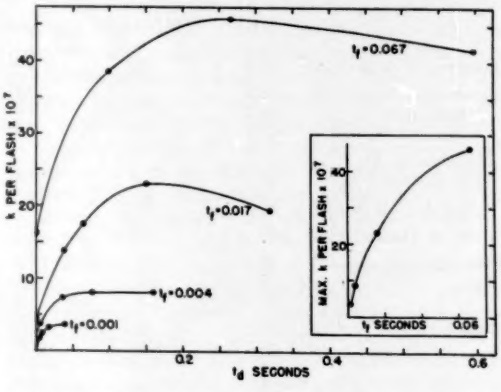

FIG. 6. Flash yield in terms of k/flash as a function of dark period for each flash time.

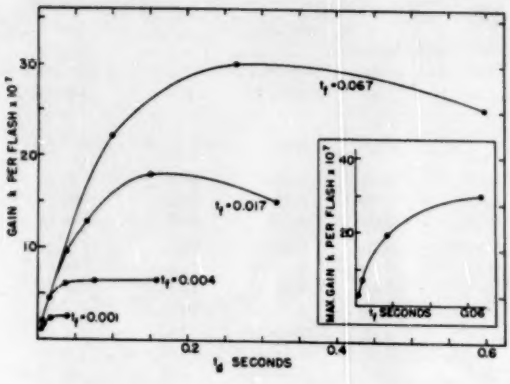

FIG. 7. Flash yield in terms of gain-k/flash as a function of dark period for each flash time. For definition of "gain-k" see text.

CO_2/O_2 has been shown to be 0.7 (4). Unless there are severe changes in nitrogen content the quotient during steady-state growth at high light intensities will be 0.7 also; at any rate it will not be higher than 0.9 as measured in short-time manometric experiments. For present purposes 224 μl carbon dioxide are taken as equivalent to 320 μl O_2 (A.Q. = 0.7) as the more likely value although it is recognized that the equivalence might be as low as 250 μl O_2 (A.Q. = 0.9). In short, values of k may be converted to μl O_2/μl cells upon multiplying by 320.

Comment should be made at this point that chlorophyll content does not enter in any way into the determination of any one value of k. Each value of k was determined during steady-state growth under conditions of constant chlorophyll content. It is true that the photometric method used is sensitive to chlorophyll absorption as well as to light scattering. However, even if the photometric device responded only to chlorophyll so that the method directly measured k in terms of Δ chlorophyll/chlorophyll per unit time, then within any one determination on a given steady-state the same value of k would describe also Δ cell number/cell number and Δ cell weight/cell weight per unit time. A quite different consequence of chlorophyll content is discussed in a later section.

The present data are compared in table IV with those obtained in other investigations. The important value of maximum flash yield shows a 27-fold variation and the minimum dark time required for maximum yield shows a 13-fold variation. Most of the variation is introduced by the longer flash times used by Tamiya (15) and in the present study and obtained by the use of rotating sectors. There are three possible interpretations. (1) Use of sectors and higher values of t_f and t_d is attended by some systematic error. (2) True flash saturation has not been achieved by different workers but is more closely approached by the use of longer flash times obtainable with sectors. (3) The use of longer flash times introduces new phenomena dependent upon different reactions from those which govern the response to very short flashes. Comment will be made on all three possibilities.

Chopping of a high intensity beam from a continuous source may give rise to serious difficulties if there is light leakage during the dark periods. In the present case the question was raised whether the higher flash yields observed with longer light flashes

TABLE IV

COMPARATIVE DATA FROM FLASHING LIGHT INVESTIGATIONS

WORKERS *	TEMP.	MAX. RATE CONTINUOUS ILLUMINATION	t_f	t_d MINIMUM FOR MAX. YIELD	t_d FOR YIELD CITED	MAX. FLASH YIELD	REFERENCE
	°C	$\mu l\ O_2/hr \cdot \mu l$	$sec \times 10^{-3}$	$sec \times 10^{-3}$	$sec \times 10^{-3}$	$\mu l\ O_2/\mu l$ per flash $\times 10^{-5}$	
Emerson & Arnold (5)	24.9	21	0.01	<35	>35	5.0	Protocols I & II
Emerson & Arnold (6)	24.9	..	0.01	..	48	10.0	Table I
high chlorophyll	24.9	37	0.01	..	83	16.0	Table II
low chlorophyll	24.9	6	0.01	..	83	3.5	Table II
Clendenning & Ehrmantraut (2)	10	..	0.01	40	100	5.2	Fig 8
Weller & Franck (16)	20	18	4.2	62	>62	13.0	Fig 2
Rieke & Gaffron (14)	20	(18) **	4.2	..	58	8.3	Table I
Tamiya (15)	25	31	8.0	150	300	40.0	Protocol IIId
	25	31	3.0	120	200	29.0	Protocol IVc
	25	31	1.0	70	200	14.0	Protocol IVb
Present data	25	28	67.0	266	266	96.0	Exp 81
	25	28	17.0	150	150	60.0	Exp 73
	25	28	4.2	40	163	23.0	Exp 66, 67
	25	28	1.0	20	41	9.6	Exp 70, 72

* The results of Kok (8) are omitted only because of lack of tabulated data.
** Value assumed equal to that observed by Weller & Franck.

could be due to a lack of integrity of the very long dark periods required. Check upon this point was made with the sector giving maximum flash yield for $t_f = 0.017$ second (experiment 73 and figure 4). From the data of figure 4 it may be seen that the middle one half of the 0.150 second dark period is entirely dark; the remaining one half includes the tails from the light flashes which summate to an average intensity equivalent to 600 erg/cm²-sec operating over 0.45 of the total time $(t_f + t_d)$. From the continuous illumination data it may be estimated that the intensity leakage in this particular regimen could not have caused an error in k of more than 0.08. A more important result, however, is that light leakage during the dark period is due only to the tails of the light flashes. The higher yields at longer light flashes and dark periods cannot be attributed to light leakage during the dark periods.

The question of attainment of flash saturation in the experiments of Emerson and Arnold (5, 6) has been examined by several workers with opposing conclusions. Tamiya (15) obtained variable t_d, t_f, and flash intensity by sector chopping of a continuous source. Maximum flash yield was obtained only with high flash energies which required long flash times and very long dark periods of about 0.2 second at 25°C. Further, the maximum flash yield was temperature dependent except at very low flash energies. Tamiya did not apply any correction for the amount of photosynthesis occurring during his long light flashes; recalculation of his data and introduction of such a correction does not make any important change in his results. Tamiya concluded that the short dark periods and other results of Emerson and Arnold were consequences of lack of attainment of flash saturation.

Weller and Franck (16) reinvestigated the question of flash saturation by use of a sector synchronized with the inherent intermittency of a mercury arc to give flashes of 0.0042 second and various dark times. Clendenning and Ehrmantraut (2) and Ehrmantraut and Rabinowitch (4a) also have presented pertinent data obtained with a neon tube flashed by a condenser discharge to give flashes of about 10^{-5} seconds similar to those of Emerson and Arnold. These studies contain various types of evidence for attainment of flash saturation in very short flashes. Their results were interpreted as supporting the argument of Emerson and Arnold that the maximum yield produced by a very short and saturating flash is determined only by the quantity of an enzyme with a turnover time of about 0.01 second at 25°C. However, no study yet made with very short flashes provides conclusive evidence for both flash saturation and temperature-independence of maximum yield which is necessary to refute Tamiya's conclusion.

A third possible explanation of the differences between short-flash and long-flash intermittency results has been suggested by Weller and Franck (16) and by Ehrmantraut and Rabinowitch (4a). When the light flash and dark period are lengthened, the limitation controlling flash yield is transferred from the "Emerson and Arnold enzyme" to some other slower enzyme system. The new system may be the one responsible for the dark pick-up phenomenon observed by McAlister (10). This is not a completely satisfying explanation. The experimental observation is that longer light flashes not only require longer dark periods, but also give higher flash yields even when the flash yield is corrected by subtracting the maximum amount of photosynthesis which could occur during the flash itself. It is a straightforward argument that lengthening of the light flash may lead to longer dark periods required by limitations of a second enzyme system. It is not at all apparent how the transfer to the second and apparently more restricting limitation can increase the flash yield.

The present data on growth rate in intermittent light have about the same precision as the manometric data of other investigations with which they have been compared. They may be compared with data on photosynthesis since the critical values for maximum flash yields and dark periods are obtained at less than the maximum growth rate where photosynthesis must be the growth-limiting process. They support the results of comparable sector experiments of Tamiya (15) and Kok (8) that long flash times require long dark periods of tenths of a second to obtain maximum flash yields. Comparison with the results of short flash experiments such as those of Emerson and Arnold (5, 6) requires either that light saturation is not attained in the short flashes or that some new limiting phenomenon is introduced by the use of longer flashes. In passing, it may be noted that none of the flashing light data support the very long dark period of the order of minutes postulated on theoretical grounds by Burk, Cornfield, and Schwartz (1).

Chlorophyll Content: Methanol extracts of the cells harvested at the end of each growth experiment showed no significant differences in the shape of the absorption curve determined between 6000 and 7000 Å. The peak of the curve at 6700 Å has been used as a measure of chlorophyll concentration. Data expressing relative chlorophyll concentration per unit volume of cells are plotted in figure 8. Chlorophyll content is the same function of light intensity previously reported by Myers (11). In intermittent light the data show the same pattern with minor deviations when plotted in terms of integrated light intensity. Unfortunately, the small quantity of cells available in each experiment limits the precision of the chlorophyll assay, and conversion to absolute amounts is not merited. Use of MacKinney's (9) absorption coefficient of 74.5 for chlorophyll in methanol at the red peak gives chlorophyll contents within the range observed by Emerson and Arnold (6).

The variability in chlorophyll concentration with light intensity means that the concentration contributes to the characteristics of the light intensity curve for growth (fig 5). The slope of the light-limited region of the curve depends upon two factors: the fractional absorption of light per cell and the quantum efficiency for growth. In analogous measurements of the light intensity curve for photosynthesis the curve is commonly determined in short-time experiments

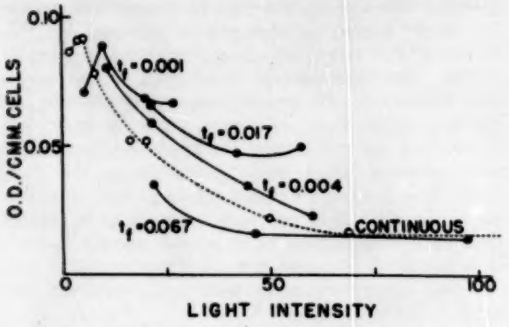

FIG. 8. Chlorophyll content of the cells as a function of light intensity at which they were grown. Relative chlorophyll content expressed in optical density per μl cells as determined at 6700 Å for a 10 ml methanol extract in a 1.0 cm cuvette. The unit of intensity is the kiloerg/cm^2-sec.

with cells of constant chlorophyll content and, hence, constant light absorption per cell. In long-time measurements of growth rates the chlorophyll content and the light absorption per cell are independent functions of light intensity. Hence any growth response to light intensity or intermittency includes the effects of intensity or intermittency upon chlorophyll concentration.

PRACTICAL ASPECTS: The entirely practical question, toward which the present work was directed, is the possible contribution of turbulence of culture to increase in growth rate in dense cultures. The light intensity of 23×10^4 erg/cm^2-sec used is about 0.6 of the maximum intensity of full sunlight in the visible region. It can be seen from the curves of figures 5 and 6 that a dense culture growing under sunlight will experience a significant increase in growth if cells are moved in and out of the high intensity of the front surface at such a rate as to give flash times between 0.001 and 0.1 second. It is also clear that the culture should be thick enough or dense enough so that almost all the light will be absorbed in the first 10 % and the dark time will be about ten times as long as the light flash. These considerations lead to the conclusion that almost any attempt to grow algae in sunlight will experience some gain by turbulence. The feasibility of increasing the turbulence will depend upon the extent of the gain in growth as compared to the increased power requirement of stirring or pumping the suspension.

SUMMARY

Growth rate of the alga, *Chlorella pyrenoidosa*, has been measured at 25°C, as a function of intensity and intermittency of illumination. The saturation curve obtained in continuous light shows a compensation point of less than 1000 erg/cm^2-sec or 24 fc and an approximate saturation point at 25×10^3 erg/cm^2-sec or 600 fc.

Intermittent light was obtained by sector chopping of a beam of 230×10^3 erg/cm^2-sec to give light flashes of 1, 4, 17, and 67 milliseconds and various dark periods. With one millisecond flashes the alga almost completely integrates intensity times time so that growth response to integrated light intensity is the same as that observed in continuous illumination. With longer flashes the degree of integration decreases but remains significant even at 67 milliseconds.

The intermittent light data have been treated also in terms of flash yield and compared with those of investigations on photosynthesis. Comparison of the critical data of the several investigations reveals a range of variation which is not explainable in terms of the classical arguments used to explain the intermittency phenomena observed with very short flashes.

The results allow prediction that partial, but probably not complete, advantage of the intermittency phenomenon may be taken to increase the efficiency of light utilization of an algal culture by turbulence of suspension.

Grateful acknowledgment is made to William A. Arnold and Bessel Kok for many helpful suggestions, and to the Carnegie Institution of Washington for aid received by the junior author as a Visiting Investigator in its Department of Plant Biology.

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RECORDING OXIDATION-REDUCTION POTENTIALS IN PLANT PREPARATIONS[1, 2]

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The study of oxidation-reduction potentials often represents a powerful tool in biological and chemical investigations (1). This paper describes simple techniques for measuring and recording oxidation-reduction potentials in photosynthetic systems. The methods described could easily be adapted to measurements on other biological and chemical systems.

Isolated chloroplasts of higher plants suspended in solutions of suitable electron acceptors (oxidants) such as ferricyanide carry on reactions of the following type upon illumination:

(1) $4 \text{ ferricyanide} + 2\,H_2O \rightarrow 4 \text{ ferrocyanide} + 4\,H^+ + O_2$

This reaction, usually called the Hill reaction (2) apparently represents the energy-absorbing and water-splitting part of photosynthesis. A number of useful techniques have been developed for studying this reaction including the measurement of oxygen evolution (2, 3), the measurement of hydrogen-ion formation (3), and the measurement of rate of decolorization of oxidation-reduction indicators (4). The Hill reaction has been studied in this laboratory by determining the rate of reduction of added electron acceptors potentiometrically (5). Advantages of this method include (a) increased precision of measurement ($\pm 1\%$), (b) system volumes of less than 0.25 ml, (c) use of oxidant concentrations as low as 10^{-6} M, (d) automatic recording of results, and (e) use of reaction cells with simple optics.

MATERIALS AND METHODS

RECORDING OF OXIDATION-REDUCTION POTENTIALS: The output of a platinum-calomel electrode system was fed through an impedance matching device into a Brown "Electronik" Single-Record Strip Chart Recorder with a self-contained amplifier. Two models were used successfully: a Series 153X11 with a pen speed of 12 seconds for full scale travel (11 inches) and a range of 0 to 2.5 mv, and a Series 153X18 with a pen speed of 1 second and a range of 0 to 1 mv. An impedance matching circuit was necessary since the input impedance of the recorder was 1000 ohms while the electrode system required a high impedance circuit to avoid polarization effects. A typical two-tube cathode follower with a bias circuit to match the tubes was used as shown in figure 1. This seemed ideal since it (a) is extremely stable, (b) shows low sensitivity to changes in plate voltage, (c) shows very low distortion, (d) has a high input impedance (estimated at 50 megohms), and (e) has a low output impedance to match the recorder. The tubes were aged before use, and stability was further increased by using low filament and plate voltages. Unsuccessful attempts were made to use an alternating cur-

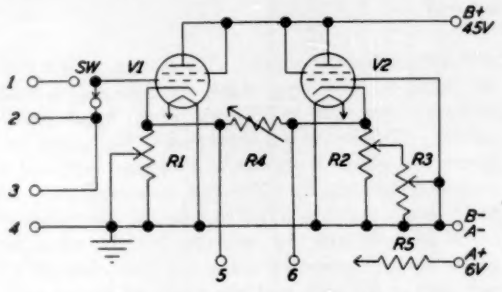

FIG. 1. Battery operated, cathode-follower, input device to match the high impedance of a platinum-calomel electrode system to the low input impedance of a Brown recorder.

V1, V2: 6K6GT radio tube.
R1, R2, R3: 10,000 ohm wire-wound potentiometer for balancing the circuit.
R4: decade resistance box to control sensitivity.
R5: 2 ohm wire-wound filament dropping resistor.
1, 2: to electrode system.
3, 4: to potentiometer for calibration and bucking voltage.
5, 6: to input of Brown recorder.
SW—SPDT: switch to connect electrode system into the input circuit.

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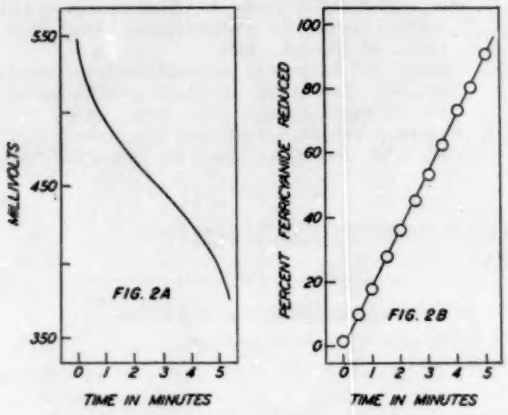

FIG. 2A. Tracing from an actual Brown recorder chart. This curve shows the results of an experiment in which red light (filter cutoff at 5800 Å) with an intensity of 8000 lux was used as a source of illumination for a Hill reaction system in a cell assembly as shown in figure 3. The reaction mixture had a total volume of 0.5 ml and contained Swiss chard chloroplast fragments equivalent to 0.1 mg of chlorophyll. The system was 0.001 M in potassium ferricyanide, 0.01 M in potassium chloride, 0.17 M in sucrose and 0.05 M in potassium phosphate buffer of pH 6.4.

FIG. 2B. Curve showing the rate of reduction of ferricyanide as derived from the potential versus time curve in figure 2A by use of a template as described in the text.

rent operated power supply. The circuit, as operated on batteries, was very stable and showed output changes of less than ± 1 % over 8-hour experimental periods. The device was connected to the Brown recorder, the electrode system, a resistance box, and a potentiometer (fig 1). The full scale sensitivity of the recorder could be adjusted from approximately 4 mv to 14 volts with the resistance box R4, while the potentiometer permitted adjusting the recorder to any part of the scale and also provided known voltages for calibration.

The potential versus time recording of a typical Hill reaction experiment is shown in figure 2A. These data may be transformed into a rate curve (percent reduction of ferricyanide versus time) by an application of the Nernst equation (5) as shown in figure 2B. Transformation was simplified by use of a slotted plastic template which, when centered on the E^o of the potential versus time recording, permitted percent reduction versus time points to be obtained directly where the recording showed through the slits.

REACTION VESSELS: The type of reaction chamber used routinely is shown in figure 3. The chamber proper consists of two pieces of transparent plastic separated by a U-shaped spacer of black opaque plastic and is clamped in an aluminum holder to permit satisfactory heat transfer. The plastic chamber is bored out at the top to hold a special calomel electrode with a pointed tip. A piece of 22 to 26 gauge wire was used for the platinum electrode since electrode area was not limiting under the conditions used. The reaction system was stirred with a piece of stainless steel wire vibrated by a small electromagnet. Stirring was essential for accurate rate measurements, especially under light-limiting conditions. All parts of the reaction system had to be illuminated to avoid non-equilibrium conditions. The principal disadvantage of the small plastic cells was the difficulty of obtaining equilibrium between the gas phase and the system because of the small surface area exposed.

To avoid this, Warburg vessels (7) were constructed of Pyrex and were provided with a calomel electrode and a platinum and/or a glass electrode as shown in figure 4. If desired, then, oxygen evolution, hydrogen ion formation, and the reduction of an oxi-

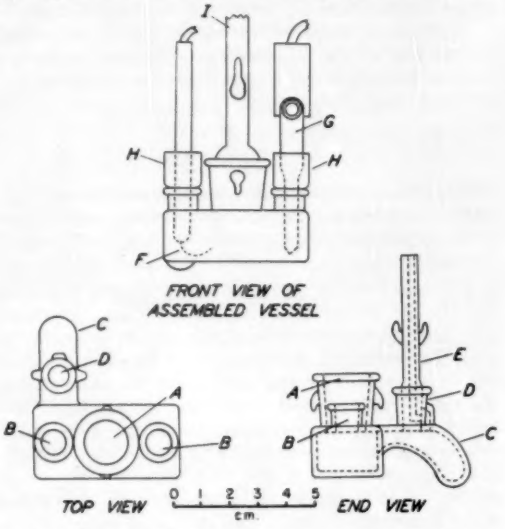

FIG. 4. Rectangular Warburg vessel modified for use in potentiometric measurements.

A—female standard-taper joint (17/20) to fit Warburg manometer or gas manifold.
B—openings for electrodes.
C—sidearm.
D—standard-taper joint for vent plug.
E—vent plug in open position.
F—platinum wire electrode.
G—calomel electrode.
H—serum bottle stopper.
I—male standard-taper joint of manometer or manifold.

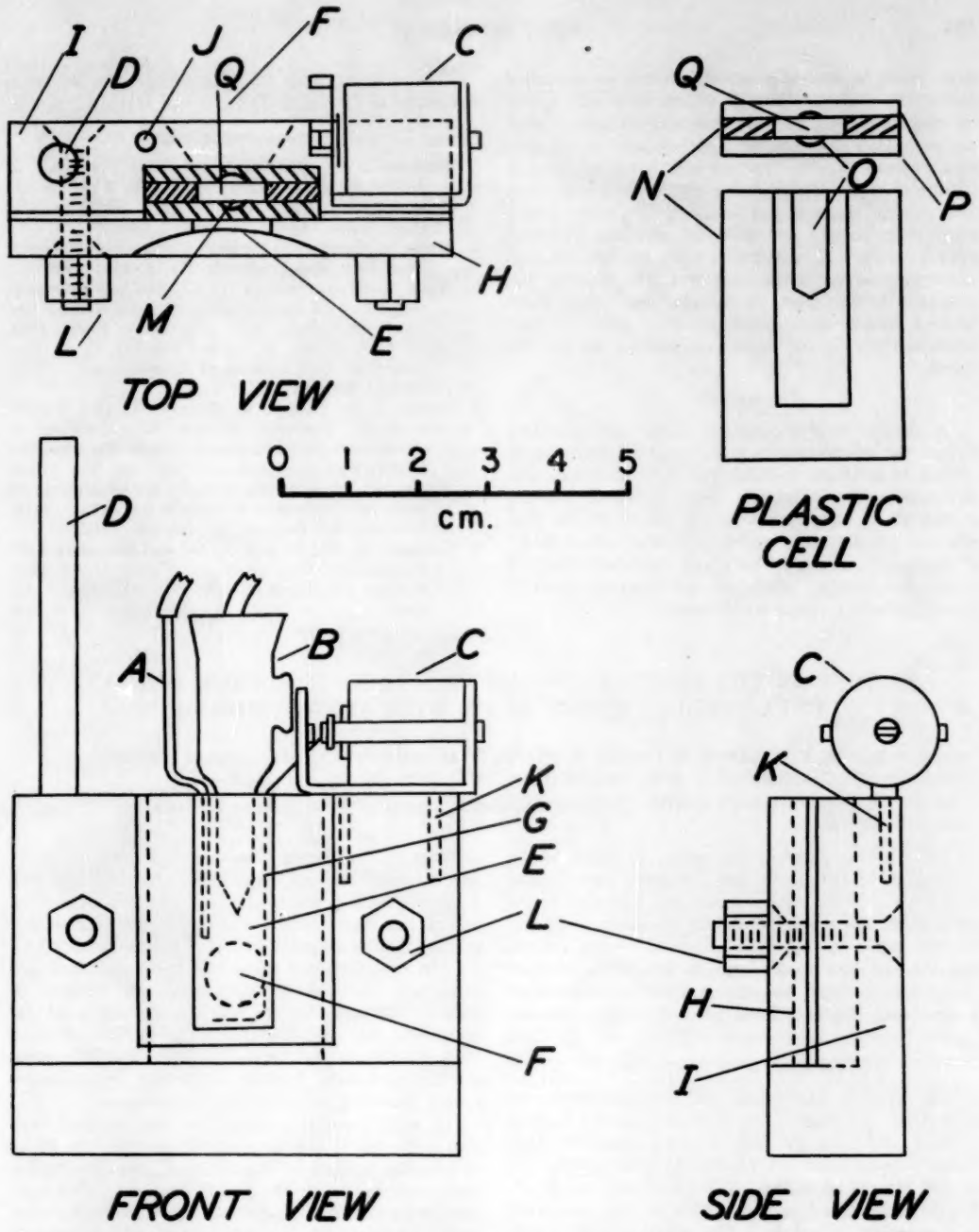

FIG. 3. Irradiation cell and associated devices for the potentiometric measurement of the photochemical activity of isolated chloroplasts.

A—platinum wire electrode.
B—calomel half-cell.
C—vibrating magnetic stirrer.
D—handle.
E—opening for light beam.
F—opening for light beam to photoelectric cell
G—wire stirrer.
H—front plate.
I—back plate.
J—well for thermocouple.
K—stirrer mounts.
L—tapered clamping nuts.
M—plastic cell assembly.
N—opaque plastic.
O—groove for calomel half-cell.
P—clear plastic.
Q—space for chloroplasts.

dant could be measured simultaneously as described earlier (6). These electrode vessels were very useful for making potentiometric measurements under different gas phases since the large surface area of the reaction mixture (3.3 cm^2 per ml with a 3.0 ml system) permitted rapid attainment of equilibrium conditions. The gassing, shaking, and temperature control procedures were carried out with the standard Warburg apparatus (6, 7). Electrodes were inserted through sleeve-type serum bottle stoppers. The sidearm was provided so that parts of the reaction system could be kept initially separated if necessary, while the vent permitted passing any desired gas mixture through the vessel.

Summary

A simple, battery-operated, impedance matching circuit for connecting a platinum-calomel electrode system to a Brown recorder for use in recording oxidation-reduction potentials from biological systems is described. Application to the study of the Hill reaction (photolysis of water by isolated chloroplasts) is discussed. Reaction cells and modified Warburg vessels for making potentiometric measurements on photosynthetic systems are discussed.

The authors wish to acknowledge the technical assistance of David H. Taysum and David C. Evans.

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STUDIES ON THE PHOTOSYNTHETIC REACTION. II. SODIUM FORMATE AND UREA FEEDING EXPERIMENTS WITH NOSTOC MUSCORUM[1, 2]

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The previous paper in this series (1) discussed the metabolism of acetate by the blue-green alga, *Nostoc muscorum*. These studies have been extended to include other low molecular weight compounds and the present paper describes the results of feeding sodium formate and urea to *Nostoc*. It will be shown that the carbon in these compounds is not assimilated to a significant degree without prior conversion to carbonate.

Materials and Methods

The growth, harvesting, and feeding procedures have been described (1). Carbon^{14}-labeled sodium formate (0.40 mc per mg obtained from the Oak Ridge National Laboratory) and C^{14}-labeled urea (61.7 μc per mg obtained from J. L. Williams and A. R. Ronzio of the Los Alamos Scientific Laboratory) were used as labeled substrates. The pre-experimental conditions and the conditions followed after addition of the tracer are described for each experiment. The light and dark portions of each experiment were carried out simultaneously on aliquots of the same algal suspension. Ten-ml aliquots were removed from the algal suspension 5, 15, 30, and 60 minutes after addition of the labeled substrate, and killed by addition to acid. In urea experiments the acid used was 4 ml of glacial acetic acid. In formate experiments, except two where 8 ml formic acid were needed as a carrier, 2 ml of concentrated H_2SO_4 were used.

The CO_2 liberated upon acidification of each aliquot was removed by subjecting the samples to vacuum (30 mm Hg) for ten minutes. In most experiments this acid-liberated CO_2 was collected in 10 % NaOH, assayed for radioactivity, and designated as the carbonate fraction. Further fractionation varied according to the type of experiment.

In most formate experiments, the samples were next subjected to high vacuum (2 mm Hg) for 48 hr to remove unchanged formic acid, the condensate being collected in dry-ice cooled receivers. The nonvolatile residues and aliquots of the condensates were oxidized and assayed for radioactivity, and designated as the assimilated and unused fractions.

In the urea feeding experiments, acidified suspensions were evaporated to dryness on aluminum planchets and assayed for total radioactivity. The urea was then hydrolyzed on the planchets by treatment with urease and the residual, non-urea C^{14} was determined after re-evaporation to dryness, suitable corrections being made for the absorption of radiation by the urease present. The loss in activity on hy-

[1] Received July 16, 1953.

[2] This work was supported by the Charles F. Kettering Foundation.

drolysis by urease is reported as urea-C^{14}, the residual activity as assimilated-C^{14}. The urease treatment was demonstrated to remove 99.9 % of the urea-C^{14}.

Recoveries of C^{14}, in their various chemical forms, are all calculated as percentages of the C^{14} added per 10 ml of algal suspension from either the urea or formate. Errors in pipetting the C^{14}-formate and C^{14}-urea occasionally resulted in recoveries of over 100 % but these were generally less significant than errors in recovering the C^{14}. In the figures, the data are plotted in an accumulated form so that the distribution of C^{14} is indicated throughout the experimental period. In this manner of plotting, the significant values are represented by vertical distances between curves rather than by direct readings on the ordinate scale.

Radioactivity Determinations: Organic residue fractions (except from experiments with urea) and formic acid fractions were converted to CO_2 by a wet oxidation procedure previously described by Skipper et al (5). The $BaCO_3$ from these oxidations and from the sodium hydroxide traps were placed on aluminum planchets (3 cm I.D.) and counted in a Nuclear Measurements Corporation proportional counter (50 % geometry). Three planchets were prepared for each sample and the average reported. Barium carbonate samples of low activity were converted to CO_2 and counted in the gas phase (5). The organic residues from urea experiments were counted directly on aluminum planchets.

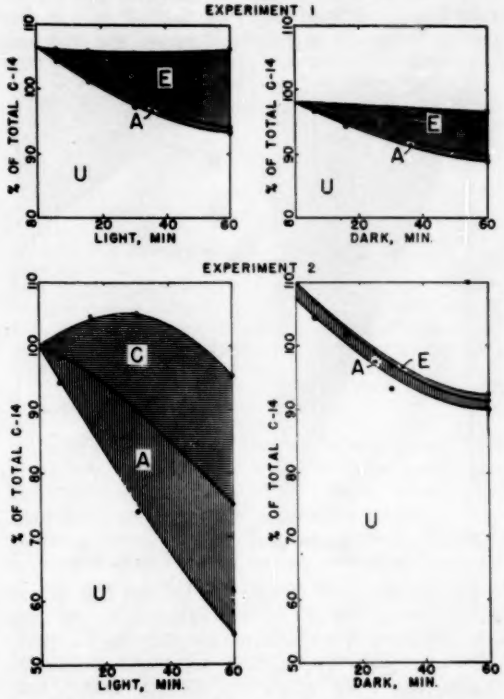

Fig. 1. The fate of urea-C^{14} fed to *Nostoc muscorum* in the light and dark. The algae were suspended in a modified Chu 10 growth medium (1) with 1.3 mg per l of $FeCl_3 \cdot 6\,H_2O$ instead of ferric citrate and citric acid. The suspension received 1 hr of pre-experimental conditioning in total darkness with 4 % CO_2-in-air aeration. In experiment 1, 4 % CO_2-in-air aeration was used during the experimental period (1 hr exposure to C^{14}-urea) and in experiment 2, nitrogen gas was employed. Code for the areas designated is: U, urea-C^{14}; A, assimilated-C^{14}; E, evolved $C^{14}O_2$; and C, carbonate-C^{14}.

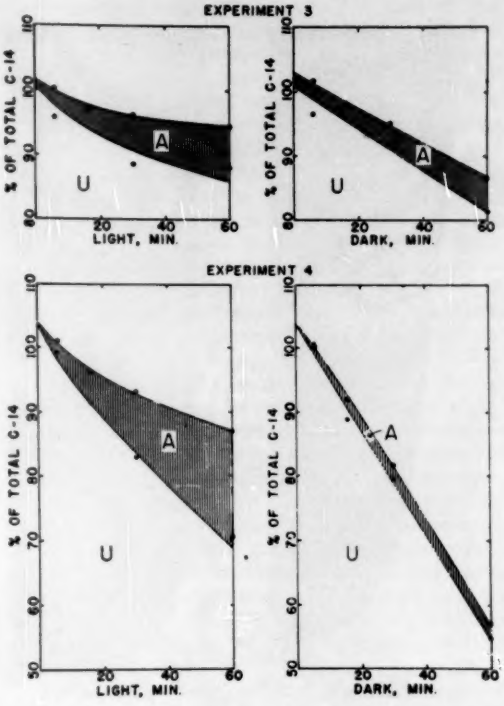

Fig. 2. The fate of urea-C^{14} fed to *Nostoc muscorum* in the light and dark. The algae were suspended in a modified Chu 10 growth medium (1) with 1.3 mg per l of $FeCl_3 \cdot 6\,H_2O$ instead of ferric citrate and citric acid, and with the Na_2CO_3 omitted. The suspension was given 1 hr of pre-experimental conditioning in total darkness with nitrogen aeration. Nitrogen gas was used during the experimental periods. Code for the areas designated is: U, urea-C^{14}; A, assimilated-C^{14}.

Chromatographic Procedure: At the conclusion of the experiments, the unused portions of the algal suspensions were filtered (with the aid of Celite). The residue on the filter was then extracted with boiling alcohol, 50 % alcohol, and water (equal volumes of each). The derived solution was evaporated and chromatographed as previously described (1).

Results

Urea-C^{14} Feeding Experiments: The results of the urea feeding experiments are presented graphically in figures 1 and 2. In none of the experiments

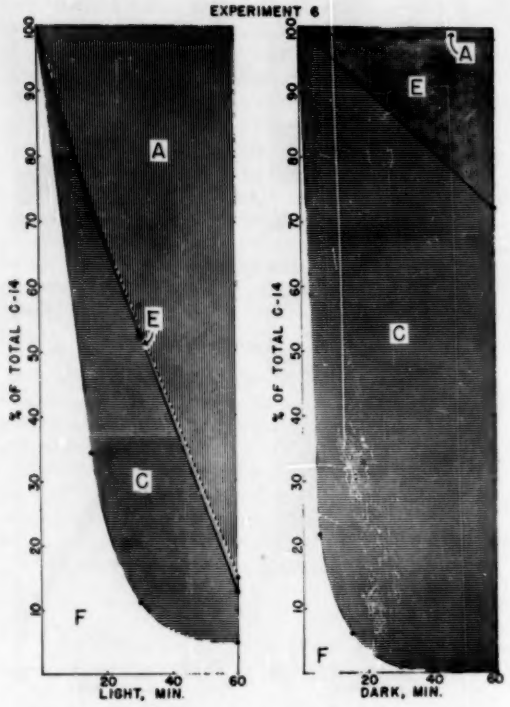

FIG. 3. Fate of formate-C^{14} fed to *Nostoc muscorum* in the light and dark. The algae were suspended in modified Chu 10 growth medium (1) with 1.3 mg per l of $FeCl_3 \cdot 6\,H_2O$ instead of ferric citrate and citric acid. Aeration with 20% oxygen in nitrogen was used throughout the 60-min pre-experimental conditioning in the dark as well as during the experimental feeding periods. Code for areas designated: F, residual formate-C^{14}; C, carbonate-C^{14}; E, evolved $C^{14}O_2$; and A, assimilated C^{14}.

did more than 50% of the urea disappear in one hour, indicating an adequate quantity of urea available for assimilation throughout the experimental period. When 4% CO_2 in air was used as an aerating gas (exp 1), the C^{14} was evolved as $C^{14}O_2$ at about the same rate that urea disappeared, and less than 1% of the total C^{14} was assimilated by the algae in either the light or dark. When nitrogen was used for the gassing (exp 2, 3, 4), 1.5 to 5.5% of the total C^{14} was assimilated in 1 hour in the dark and up to 20% in the light. The large fixation of urea-C^{14} in the light when the carbonate-C^{14} was left in the medium (exp 2, 3, 4) and the slight fixation when the carbonate-C^{14} was removed as $C^{14}O_2$ (exp 1) demonstrate that very little, if any, urea was assimilated as such, and that the assimilation proceeded via carbonate (or CO_2). Even in the dark, the C^{14} assimilation was greater when $C^{14}O_2$ was not removed efficiently (N_2 gassing).

Chromatograms provided reasonable confirmation that only $C^{14}O_2$ was assimilated when C^{14}-urea was fed to *Nostoc*. Chromatograms and autoradiograms prepared from the urea experiments showed C^{14} distribution patterns of metabolites very similar to those obtained following radiocarbonate-feeding. Unusually high activity was found in glutamine and asparagine while low activities (compared to $C^{14}O_2$-feeding) occurred in glutamic acid and aspartic acid. Labeled urea was found as well as one heavily labeled unidentified compound which traveled close to urea on the paper chromatograms. The activity in histidine, arginine, or citruline (which might have been derived directly from urea) was not unusually high.

FORMATE-C^{14} FEEDING EXPERIMENTS: The utilization of formate-C^{14} by *Nostoc* is shown graphically in figures 3 and 4. These data suggest the mechanism of formate utilization by demonstrating a large assimilation of formate-C^{14} aerobically in the light (fig 3) and the greatly reduced assimilation in the dark (fig 3) and anaerobically in the light (fig 4).

The latter effect was better illustrated in experiment 5 which differed from experiment 6 only in that gassing during the experimental period was with just

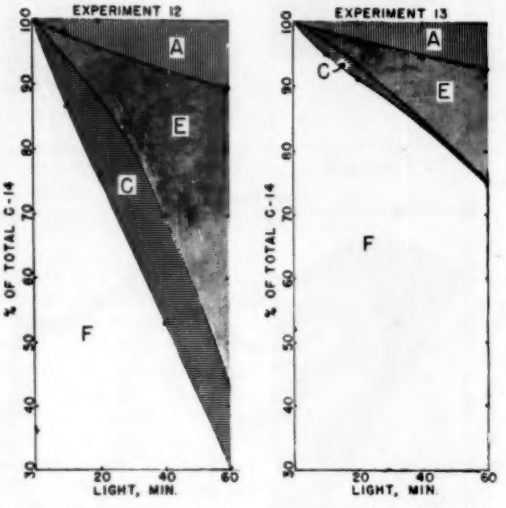

FIG. 4. Effect of aerating gas on the fate of formate-C^{14} fed to *Nostoc muscorum* in the light. The algae were suspended in the following medium (mg per liter): $Ca(NO_3)_2 \cdot 4\,H_2O$, 57.6; K_2HPO_4, 10; $MnSO_4 \cdot 7\,H_2O$, 25; Na_2CO_3, 265; $FeCl_3 \cdot 6\,H_2O$, 1.3; $Na_2B_4O_7 \cdot 10\,H_2O$, 0.88; $MnCl_2 \cdot 4\,H_2O$, 0.36; $ZnCl_2$, 0.021; $CuCl_2 \cdot 2\,H_2O$, 0.027; $Na_2MoO_4 \cdot 2\,H_2O$, 0.25; $CoCl_2 \cdot 6\,H_2O$, 0.04. When used in conjunction with 1% CO_2 in air this nutrient has a pH of about 7.5 and permits very rapid growth of *Nostoc*. In experiment 12, the algae were aerated with 1% CO_2 in air. In experiment 13 the 1% CO_2 was in nitrogen.

The pre-experimental conditioning was 5 minutes' total darkness followed by 5 minutes of light. Code for the areas designated is: F, residual formate-C^{14}; C, carbonate-C^{14}; E, evolved $C^{14}O_2$; and A, assimilated-C^{14}.

nitrogen.' The one hour assimilation in light was 1.75 % compared to the 80.5 % indicated in figure 3.

A separate indication of the assimilation mechanism is to be noted in the rate at which formate is converted to carbonate. In figure 3 this reaction is seen to proceed to completion so rapidly that it could hardly be considered a reaction competitive with the observed assimilation. It must instead be a step within the assimilation mechanism.

In experiments 12 and 13 the identity of the unused C^{14}-formate was established by isolating this fraction as a condensate from vacuum drying (1 mm Hg) of algal samples which had been killed with carrier formic acid. Duclaux values were determined on the condensate and were compared with "C^{14}-Duclaux values" determined simultaneously. The results were in sufficient agreement so that only average values for the 9 condensate samples analyzed are reported in table I. It can be seen that the C^{14} values are in very good agreement with the titrated values and with reported formic acid Duclaux values and are

TABLE I

IDENTIFICATION OF UNUSED C^{14}-FORMATE. DETERMINATION OF "C^{14}-DUCLAUX VALUES" ON CONDENSATE SAMPLES

FOR DISTILLATE SAMPLE	DUCLAUX VALUES *		
	1	2	3
Acetic acid, from literature	6.8	7.1	7.4
Formic acid, from literature	3.95	4.40	4.55
Experimental av. on 9 samples: **			
By titration	3.43	4.28	4.80
By C^{14} distribution	3.82	4.31	4.95

* These values are determined on three consecutive 10 ml distillate samples from 100 ml of each condensate solution containing 2 % carrier formic acid.

** Of the condensate samples analyzed seven were from experiment 12 and two from 13.

quite different from reported acetic acid values. The $C^{14}O_2$ was thus identified with the carrier formic acid and only very small amounts of other C^{14}-compounds were likely present in the condensate samples.

In chromatographic study of extracts of *Nostoc* which had been fed formate and carbonate, no significant differences could be found in C^{14} distribution in metabolites for duplicate experimental conditions. When air was used as the aerating gas the formate-C^{14} was found widely distributed in the products normal to C^{14} assimilation. When CO_2 was employed in the aerating gas C^{14} was found in fewer compounds with relatively greater concentrations in sucrose and some pigments.

DISCUSSION

This study of urea utilization by *Nostoc* has been confined to urea-carbon utilization whereas most urea studies in plants have been concerned with nitrogen metabolism. Hinsvark, Wittwer, and Tukey (2) studied the fate of foliar-applied urea in plants and concluded that the urea was first hydrolyzed by urease giving NH_3 and CO_2. Similar conclusions can be drawn from our experiments where it was shown that the radioactivity on the paper chromatograms was found in compounds normally labeled in $C^{14}O_2$ photosynthesis. Walker (6) could not demonstrate urease activity in *Chlorella* and postulated that urea might enter the metabolic pool by means of a reversal of the mammalian, mold urea cycle. If this had been true with *Nostoc*, one would have expected that arginine or citrulline would have been heavily labeled by feeding C^{14}-urea but this was not found to be true. Urea assimilation rate studies provide additional evidence that the urea-C^{14} was not assimilated to an appreciable extent without prior conversion to carbonate. It can only be concluded that *Nostoc* hydrolyzed the urea (presumably with urease) and assimilated the products (ammonium and carbonate ions).

In the feeding of C^{14}-labeled formate to *Nostoc muscorum* it has been shown that the rate of radiocarbon assimilation was dependent upon the concentration of C^{14}-labeled carbonate (derived from the formate) available to the algae. Under anaerobic conditions much less formate was consumed, less C^{14}-carbonate was available, and less C^{14} was assimilated. Under aerobic conditions most of the formate disappeared and much of the C^{14} appeared as carbonate, either to be assimilated or be swept from the medium by CO_2 aeration. The C^{14} assimilated aerobically in the light was found in products normally labeled in $C^{14}O_2$ photosynthesis.

These data, then, indicate that the principal mechanism for assimilation of formate carbon is through its oxidation, in the presence of algae, to carbonate. Similar conclusions were reached by Mosbach et al (4) who were studying the incorporation of formate into the citric acid formed by *Aspergillus niger*. Mathews and Vennesland (3) found that many plant and animal tissues contained the enzyme, formic dehydrogenase, which mediated the oxidation of formic acid to carbon dioxide. We conclude that direct photosynthetic fixation of formate by *Nostoc muscorum* is improbable or insignificant.

SUMMARY

Urea and formate are converted to carbonate by *Nostoc muscorum* by hydrolysis and oxidation, respectively, and the resulting carbonate is assimilated in the normal manner. Neither substance is assimilated to a significant degree without conversion to carbonate and neither has a significant role in the photosynthetic process.

The authors wish to thank Miss Linda Simpson for assistance in making the radioactive determinations in this work and A. J. Tomisek and G. R. Noggle for suggestions in the preparation of the manuscript.

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FRUIT RESPIRATION AND ETHYLENE PRODUCTION[1]

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The primary objective of this study was to determine the causal relationship between ethylene production and the onset of the climacteric rise in respiration. The marked rise in oxygen uptake and carbon dioxide output, known to fruit physiologists as the "climacteric" rise, is a characteristic phenomenon of the ripening process. It marks a transition phase between development and the onset of functional breakdown, between ontogeny and senescence. The physiological and biochemical changes associated with the climacteric were reviewed recently by Biale (3), and the role of ethylene in fruit storage was summarized by Porritt (18).

One of the intriguing problems in fruit ripening is the question of whether ethylene induces the rise in respiration or is a product of this rise. In practically all cases ethylene was found to accelerate the onset of the climacteric if applied before the rise. Responses from external applications of ethylene are by themselves insufficient evidence that the naturally occurring climacteric is a result of the ethylene produced by the fruit. The information available on the relationship between ethylene production and respiration is limited and conflicting. Nelson (16) found that the sharp rise in ethylene production of McIntosh apples followed the rise in CO_2 evolution. In the case of bananas Nelson (15) observed an inverse relationship between ethylene and CO_2 evolution. His samples of both apples and bananas were on the climacteric rise at the start of the experiments. Hansen (8), working with pears, observed that the maxima in both processes occurred at the same time, but the relative increases calculated as the ratio of maximum rate to initial rate were much lower for respiration than for ethylene production. His data point to a close parallelism between the onset of the rise in CO_2 and in ethylene. A similar behavior was obtained by Pratt and Biale (19) for avocados. These authors employed a biological assay which at best is semi-quantitative. Since the initial values for ethylene production are intrinsic to this problem, a reliable and specific quantitative method is essential. The manometric technique developed in this laboratory (21) made it possible to obtain quantitative data on the avocado and to extend information on ethylene production to other fruits about which no data were available. This report includes also, for the first time, evidence for the occurrence of the climacteric in several tropical and subtropical fruits. For purposes of comparison we included studies on a few of the temperate zone fruits, although the more extensive investigations were concerned with fruits from tropical and subtropical regions.

[1] Received July 22, 1953.

Materials and Methods

The fruit samples of the avocado (*Persea* sp.), Valencia orange (*Citrus sinensis*), sapote (*Casimiroa edulis*), and feijoa (*Feijoa sellowiana*) were obtained from the departmental orchard and placed under experimentation immediately after harvesting. The Navel oranges (*Citrus sinensis*), lemons (*Citrus limon*), and persimmons (*Diospyros kaki*) were received directly from a packing house. The cherimoyas (*Annona cherimola*) were grown in a commercial orchard in San Diego County and used within two days after picking. The mangos (*Mangifera indica*) were shipped from Florida by air express and were placed in the constant temperature chamber within two to three days from harvest time.[2] The bananas (*Musa sapientum*) originated in Central America, where they were picked "¾ full," shipped by refrigeration, and arrived in California in a fully green condition. The state of maturity of the pineapple (*Ananas camosus*) from Cuba and of the papaya (*Carica papaya*) fruit from the Hawaiian Islands was not as satisfactory as that of the other fruits. The results obtained with pineapple and papaya indicate only whether ethylene is produced in the ripe fruit, but they throw no light on the main objective of this study. The temperate zone fruits were either procured on the wholesale market or shipped from the University Farm at Davis.[3]

The methods employed consisted of measuring respiration by carbon dioxide evolution, and ethylene production by the manometric technique. A stream of air was passed at a constant rate through a flowmeter, over a jar of fruit, and then into a cylinder

[2] We wish to express our thanks to Miss Margaret Mustard of the University of Miami for procuring and shipping the mangos used in these investigations.

[3] We are indebted to Dr. L. L. Claypool for the Bosc pears and to Dr. A. M. Kofranek for the McIntosh apples.

containing alkali for CO_2 absorption, or into an absorber with mercuric perchlorate for ethylene absorption. Respiration measurements were made over a period of two to four hours each day, while ethylene was absorbed for a period of 20 to 22 hours. The ethylene method was developed in this laboratory and has recently been reported in full (21). Briefly it is based on the fact that ethylene forms a complex with mercuric perchlorate. The accumulated ethylene is released from the complex by the addition of hydrochloric acid, and its volume is measured manometrically. With this method 0.2 ml of ethylene can be determined with an accuracy of 5 %. This value is equivalent to 0.33 ppm of ethylene in an air stream of 200 ml/min for a period of 50 hours. It is possible to measure smaller concentrations by using longer periods of time or by determining the gas evolved to a lower degree of accuracy.

Results

The Avocado, *Persea americana* × *Persea drymifolia:* The Fuerte variety used in these studies is considered a cross between a Mexican and a Guatemalan race of avocado. Its season of maturation is relatively long. Fruits developed from the January to April bloom attain their maximum fat content of 15 to 25 % (on fresh weight basis) in March of the following year. By that time sugars have declined to a value of 0.25 to 0.50 % and the protein content has reached 2.0 to 2.5 % of the fresh weight. The avocado is, then, a characteristically high fat, high protein, and low carbohydrate fruit.

In southern California coastal districts, fruits of the Fuerte variety may be harvested from October to August, though the commercial harvesting season is normally over by June. Fruit softening will not take place while the fruit is attached to the tree and while the stem is healthy. However, immediately after harvesting the processes leading to senescence are set into action and the typical climacteric course of respiration ensues, as shown in figure 1 for fruit placed at 20°C, and in figure 2 for fruit at 15°C. Late season Fuerte avocados reach the climacteric peak sooner and exhibit a sharper rise than early season fruit, but the actual values for CO_2 production at the peak remain approximately constant throughout the season and are a function of the storage temperature and of the atmospheric composition of the storage environment. At 20°C the rate of ethylene production follows closely the rate of respiration during the course of the climacteric, but no measurable quantities of ethylene were detected prior to the onset of the rise in CO_2 output. After the climacteric peak the rate of ethylene evolution decreased more markedly than that of CO_2 production. At 5°C no pronounced climacteric rise was observed and no significant ethylene values were determined. It should be noted that upon transfer from 5°C to 20°C after 34 and after 43 days, there was a rise in CO_2 output with no corresponding rise in ethylene evolution. This difference in the response of the CO_2 and of the ethylene reactions might serve as a basis for a new approach to the widely perplexing problem of the effects of relatively low temperatures on the ripening processes in fruits and vegetables. It is known that prolonged exposure of a number of avocado varieties to 5°C or lower will cause physiological disorders. Discoloration of the flesh and the skin occurs after removal to higher temperatures and frequently normal fruit softening is inhibited. Apparently the suppression of the measurable climacteric rise at 5°C is responsible for the observed symptoms. However, one cannot exclude the possibility that certain physiological changes do occur at 5°C which are similar to those determined at higher temperatures. Pratt and Biale (19) reported the production of an active emanation by Fuerte avocados after exposure to 5°C for 57 days. They identified this emanation as ethylene by the triple response of pea seedlings. The concentration evolved was approximately 0.5 ppm. They obtained no pea response during the first eight weeks at 5°C, as was the case in this study.

The experiments with avocados at 5°C and at 20°C brought out several important points on the relation of ethylene to respiration, but failed to show whether ethylene induced the rise or was a result of it. The changes at 20°C were too rapid to show the causal relationship. We thought then that the responses at 15°C might be sufficiently slow to allow measurements in the initial stages of the rise. The results of one of the studies at 15°C are presented in figure 2.

It is evident from this figure that the rise in ethylene evolution started about the same time as the rise in CO_2 production. The ratios of peak to initial values are much higher for ethylene than for CO_2. The rate of decrease after the peak is also considerably greater for ethylene than for respiration. While the actual magnitudes are lower at 15°C than at 20°C, the general behavior was essentially the same under those two conditions. The evidence from avocados neither supported nor refuted the idea of an ethylene induced climacteric. We turned our attention, therefore, to other tropical fruits.

The Banana, *Musa sapientum:* The banana, unlike the avocado, is a high carbohydrate, low fat fruit. Changes occurring in bananas during ripening were described in a recently published monograph by von Loesecke (11). In the course of fruit ripening, starch decreases from about 20 to 1 % and sugars increase from about 1 to 18 %. The nitrogen content in the edible portion of the ripe banana amounts to about 0.2 % of the fresh weight. Applied ethylene accelerates the chemical changes associated with ripening and causes a shift in the time axis of the onset of the climacteric rise. The question before us was whether the normally occurring ripening changes are brought about by ethylene produced by the fruit. A qualitative identification of ethylene in the gaseous emanations of bananas was reported by Niederl et al (17). We made a concurrent quantitative study of ethylene production and CO_2 evolution at 15°, 20° and 25°C. The results at 20°C are shown in figure 3.

The first significant value in ethylene evolution was observed on the fifth day and it corresponded with

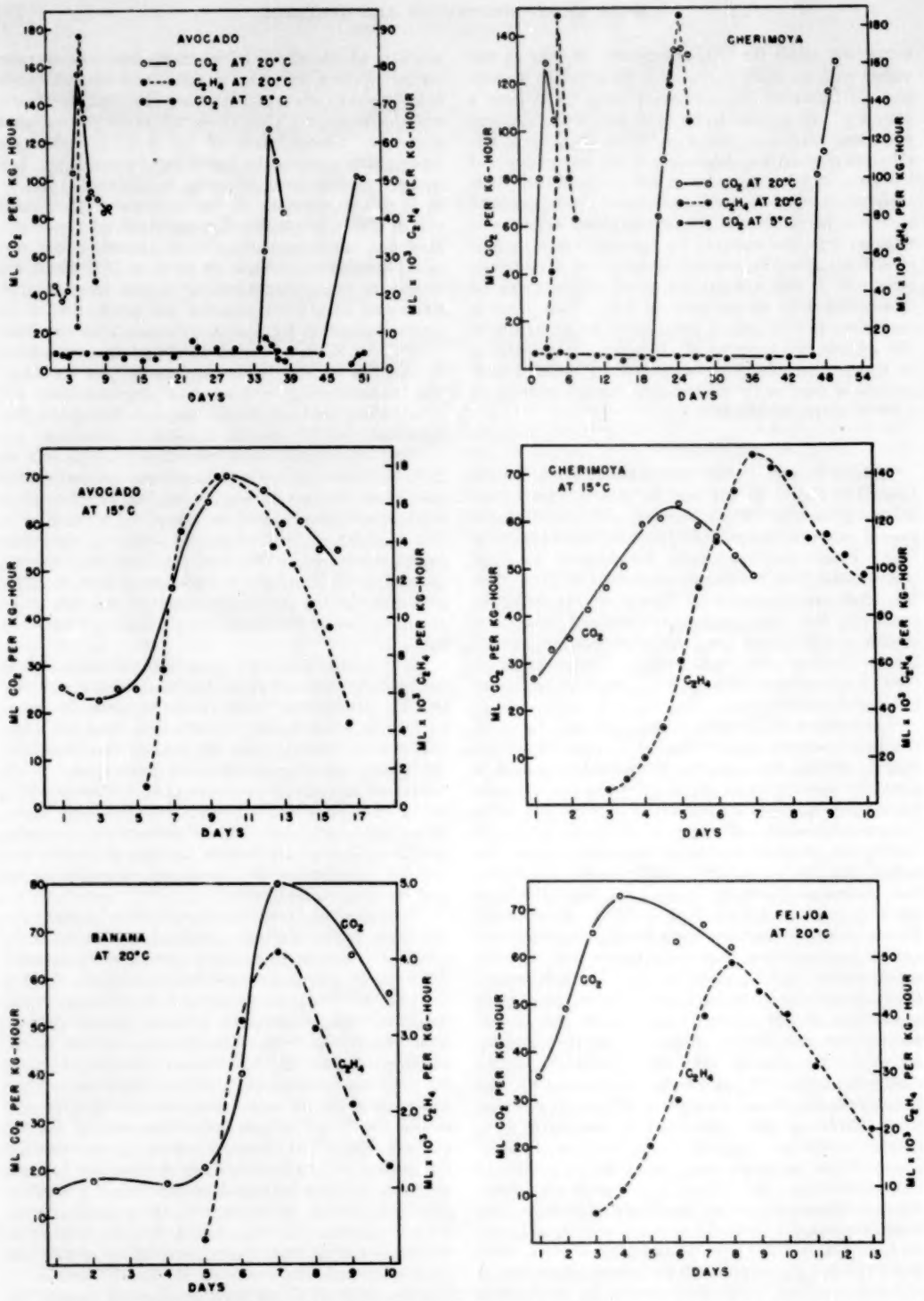

FIG. 1 to 6. Fruit respiration and ethylene production (solid line CO_2, broken line C_2H_4). Fig. 1, 2—Avocado. Fig. 3—Banana. Fig. 4, 5—Cherimoya. Fig. 6—Feijoa.

the onset of the respiratory rise. While a similar curve was obtained for 15°C, the ethylene values were too low to be conclusive. Less than two μl of ethylene were produced by one kg of bananas for one hour during the peak of activity at 15°C. At 25°C the rate was four times higher than at 15°C, but the relationship between ethylene production and respiration was essentially the same as at 20°C. We have seen, therefore, that in the banana as in the avocado no clear conclusion can be drawn as to whether ethylene production precedes or follows the onset of the respiratory rise. We proceeded to extend our survey to fruits of lesser economic importance than those discussed up to this point.

THE CHERIMOYA, *Annona cherimola:* The cherimoya is considered to have originated in the highlands of tropical South America. It is grown on a very limited scale commercially. In describing this fruit Chandler (6) states that "at its best the custard-like flesh has a pleasant blend of sweetness and mild acidity combined with flavors suggestive of the pineapple or the nectarine, but a richness all its own." The ripe fruit is high in sugars (18 % on fresh weight), high in proteins (1.8 %), and low in acid content (0.06 %). The Booth variety used for this study varied in size from 300 to 400 gm per fruit. The course of respiration and of ethylene production at 20°C and at 5°C is shown in figure 4.

The sharp rise in ethylene production associated with the climacteric at 20°C suggests a general behavior similar to the avocado and the banana. There appears to be, however, an important difference. The onset of the rise in ethylene production is definitely later than the beginning of the rise in CO_2 evolution. This is reasonably clear at 20°C but definitely convincing at 15°C (fig 5). At 5°C no climacteric rise in respiration and no significant ethylene production were observed for 46 days.

The transfer from 5°C to 20°C after 20 and 45 days caused a rise in CO_2 evolution in both cases. However, the rise in ethylene production took place only in fruit transferred to the higher temperature after 20 days' storage at 5°C but not after 45 days. Apparently here, as in the avocado, a prolonged exposure to 5°C affected the ethylene reaction more markedly than the overall respiratory process. The cherimoya is doubtless subject to chilling injuries like most other fruits, but we are unaware of any study dealing with this problem. We do not have, therefore, sufficient information on the cherimoya to suggest an explanation for the difference between the first and the second transfer.

The evidence obtained from this fruit suggests that ethylene is a product of the respiratory changes peculiar to senescence rather than a causal agent. This observation made it necessary to extend the work to other fruits.

THE FEIJOA, *Feijoa sellowiana:* The feijoa, also known as the pineapple guava, is perhaps the least tropical of the fruits discussed thus far. It is more resistant to low winter temperatures than either the avocado or the cherimoya, and it tends to produce a higher quality fruit in districts with cool summers. The fruit is relatively low in sugars (6 to 8 % of fresh weight), average in proteins (0.8 %) and rather low in acid. It is not suitable for shipment because of rapid deterioration of the central portion.

For this study the Coolidge variety was used. On account of the green color of the skin the state of maturity at harvesting could not be determined with precision. However, the sample appeared to be reasonably uniform if one may conclude from the regular respiratory behavior as shown in figure 6. Here, as in the cherimoya, the rise in ethylene followed the rise in respiration.

THE MANGO, *Mangifera indica:* For this study the Haden, Fascell, and Brooks varieties of mango were used. The fruit was relatively green when received. The immature mango has a considerable starch content which, upon ripening, changes into sugar. The ripe mango has a rather high sugar (15 %) and high acid (0.5 %) content, and is characterized by a turpentine-like flavor due to aromatic substances.

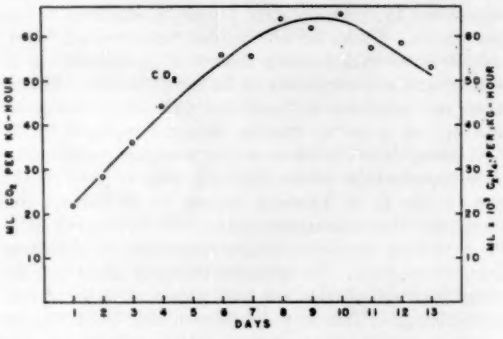

FIG. 7. Carbon dioxide production by Haden mango at 20°C.

Four experiments carried out with the mango gave essentially the same results. The data from one test with Haden are indicated in figure 7. Here we see a typical climacteric rise in respiration, but at no time were we able to detect measurable quantities of ethylene. The ripening process was normal with characteristic skin colors developing during the climacteric cycle. This is then a striking case with a typical climacteric unaccompanied by ethylene evolution into the gas phase. In one case a sample of fruit subjected to ethylene did show an accelerated rate of respiration. The ability of the fruit to respond to external ethylene is not correlated with its capacity for producing ethylene. A similar response was observed with citrus fruits, which are affected by ethylene but do not produce it.

THE ETHYLENE STORY IN CITRUS: The literature is replete with conflicting evidence concerning the problem of ethylene production by citrus fruits. Miller et al (14) reported that sound oranges caused epinasty of tomato plants when enclosed in a sealed jar. They also observed that fruit inoculated with the

common green mold, *Penicillium digitatum*, brought about an epinastic response. At the same time Biale (2) found that an active emanation, presumably ethylene, was produced by this fungus when grown on fruit or on agar medium. This active emanation was identified as ethylene by Young et al (20). At no time was there evidence published from this laboratory which would support the contention that citrus fruits free of mold produce ethylene. We were, therefore, incorrectly quoted by Miller (13), Von Loesecke (11), and by Bartholomew and Sinclair (1). On the basis of our experiences we consider the epinastic response in a closed system as insufficient evidence for the identification of ethylene.

Recently we carried on several studies with lemons and oranges in an attempt to settle this question. There was no response of pea seedlings to emanations of fruit kept in air at 15°, 20°, and 25°C. A response was obtained in some instances when fruit was placed under 100% oxygen. With the manometric method we obtained a maximum of 56 μl per kg of Valencia oranges in 24 hours under pure oxygen. This was equivalent to approximately 1 ppm of ethylene in the gas stream. Under air no ethylene was detected. Our studies were in a moving stream as contrasted to a still atmosphere employed by Miller et al (14). Moreover, our evidence is based not only on a biological test but on a rather specific chemical method. Hall (7), using direct absorption in potassium permanganate, reported the production of five to six μl of ethylene by one kg of Valencia oranges in 96 hours. He found also that cut segments and fruit inoculated with *P. digitatum* produced larger quantities of ethylene than intact fruit. We question seriously these and all other findings of Hall in this paper on grounds of methodology. The only evidence which he brings in support of the permanganate method is a comparison with the mercuric perchlorate absorption technique. He carried out this comparison on two different varieties of apples. He realized full well the great varietal differences, and he should have realized the properties of permanganate to react also with volatiles other than ethylene. Nelson (15), who was perhaps the first to use the permanganate method, took the precaution of absorbing the nonethylenic volatiles by sodamide, but there is no evidence that Hall (7) did. It is our contention that any proof for the production of ethylene by citrus fruits has to be based not only on the use of the proper methods but must be accompanied by convincing evidence that the sample was at all times free of ethylene-producing molds.

Comparative Rates of Ethylene Production: Table I summarizes the results obtained with the fruits discussed up to this point and includes findings for several additional fruits. Wherever a definite climacteric peak was observed the maxima reported correspond to the values for the peak. In the case of the sapote, the rise of ethylene production appeared to start three to four days later than the rise in CO_2 output at 20°C. This could not be ascertained with precision because the samples of sapote were not as uniformly mature as most of the other fruits. Our evidence is not clear-cut about the occurrence of the climacteric in the persimmon. There is no question that the citrus fruits do not exhibit the climacteric rise at ordinary temperatures and under normal atmospheric conditions. On the other hand, a respiratory rise was reported by Biale and Young (4) for lemons subjected to oxygen levels higher than air. It was not clear, however, that this rise was the typical climacteric rise, since the citrus fruits apparently do not undergo chemical changes unique to the ripening

Table I

Concurrent Values for Ethylene and Carbon Dioxide Production

Fruit	Variety	Temp. °C	C_2H_4 ml/kg · hr × 10^5	CO_2 ml/kg · hr	$\frac{C_2H_4}{CO_2} \times 10^5$
Tropical					
Banana	Gros Michel	20	4	80	0.05
Mango	Haden	20	0	65	0.0
Papaya	.....	25	37	44	0.84
Pineapple	.....	25	0	42	0.0
Subtropical					
Avocado	Fuerte	20	88	156	0.56
Cherimoya	Booth	20	186	129	1.44
Feijoa	Coolidge	20	50	73	0.69
Lemon	Eureka	25	0	6	0.0
Orange	Valencia	25	0	8	0.0
Orange	W. Navel	20	0	8	0.0
Persimmon	Hachiya	20	2	17	0.12
Sapote	Pike	20	129	43	3.0
Temperate					
Apple	McIntosh	20	112	12	9.3
Pear	Bartlett	20	122	42	2.9
Pear	Bosc	20	29	14	2.1
Peach	Hale	20	36	37	0.97

process in other fruits. With the pineapple, we observed no respiratory rise but our experiments were too few to be conclusive.

The last column in table I was designed to be used for comparing the ethylene activity values in relation to the respiratory capacities of the various fruits. Generally a low rate of CO_2 production is associated with low or no ethylene evolution. The McIntosh apple is a notable exception with an unusually high rate of ethylene evolution. Our findings with this variety agree with those of Nelson (16). There seems to be also some correlation between high rates of ethylene production and high rates of respiration. Of the fruits which produce ethylene the banana is the only exception to this direct correlation. It is interesting to note that the temperate zone fruits show a high ethylene to CO_2 ratio. Our results for Bartlett pears correspond closely with those of Hansen (8), who used the micro-bromination method. Compared to the Bartlett, the rate of ethylene evolution of the Bosc pear was reduced to about the same extent as the rate of respiration. It would be of interest to have data on other varieties and species of temperate zone fruits. The information available for all the species reported here suggests, however, that the ethylene-producing mechanism might be affected by factors which leave the overall respiration intact.

Discussion of Results

The fruits under discussion in this paper might be classified into the following three groups with respect to the relation of ethylene production to respiration.

(A) Fruits with a pronounced climacteric capable of producing significant quantities of ethylene. Examples: avocado, banana, cherimoya, feijoa, sapote.

(B) Fruits with a pronounced climacteric but devoid of ethylene emanation. Example: mango.

(C) Fruits not exhibiting a climacteric and not producing ethylene under ordinary conditions. Example: lemon and orange.

We do not know of any case in which fruit fails to exhibit a climacteric under any ripening temperature but does produce physiologically important quantities of ethylene. The minimum external concentration of this gas required to accelerate the ripening process is probably of the order of 0.1 ppm, though conclusive evidence on this point is lacking. We do not know the relation between external and internal concentration in the fruit tissue. Until this is established, one can advance the argument that small quantities sufficient to induce ripening are produced prior to the rise of respiration, but measurable amounts are detected only after the onset of the climacteric. Hansen (9) working with premature pears postulated the hypothesis that when the concentration of ethylene is sufficiently high the climacteric rise ensues. His results show, however, a close parallelism between the increase in CO_2 and ethylene production, similar to our findings with the avocado and the banana. The delay in the onset of the climacteric in a highly aerated system can be explained in terms of ethylene dilution, but the evidence is certainly indirect. The findings of Kidd and West (10) have some bearing on the question of the induction of the respiratory rise. They subjected pre-climacteric apples to ethylene for different exposure periods. The fruit treated for one or five hours exhibited temporary stimulation which was soon followed by recovery to the level of the controls. The climacteric did not take place as a result of stimulation. The action of ethylene as an inducing agent was insufficient. It was necessary to maintain the ethylene application in order to continue the respiratory rise. We think that this study of Kidd and West supports the idea advanced here that ethylene is not necessarily the primary causal agent for the onset of the climacteric when normal ripening takes place. Changes in metabolic reactions of a more universal nature are presumably associated with the chemical transformations characteristic of the climacteric. Some attempts were made to describe these reactions (5, 12), and more studies are in progress dealing with oxidative phosphorylation of the cytoplasmic particles obtained from fruit at different stages of the climacteric cycle.

Summary

1. Fourteen species of fruits of tropical, subtropical, and temperate climatic zones were investigated for the relationship between ethylene production and respiration.

2. The occurrence of the climacteric rise in CO_2 production was established in several species and confirmed in others.

3. The fruits with a marked climacteric showed appreciable to high rates of ethylene production with the mango as the sole exception.

4. The ratio of ethylene evolution to CO_2 output was the highest for the apple, followed by the sapote, pear, cherimoya, peach, papaya, feijoa, avocado, persimmon, and banana.

5. Oranges and lemons exhibited no climacteric in air and produced no ethylene.

6. The hypothesis was advanced that native ethylene is a product of the ripening process rather than a causal agent.

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THE EFFECT OF X-RAYS ON UPTAKE AND LOSS OF IONS BY POTATO TUBER TISSUE[1]

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Alteration in permeability has frequently been cited as an effect of x-rays on cells (1, 2, 7). X-rays have been found to induce changes in permeability, both increased and decreased permeability having been reported in various plant and animal tissues (1). Since it is now well known that ion uptake or loss in living cells is largely regulated by metabolic activities, such changes may well be referable to alterations in an active transfer or transport system. The present investigation was undertaken to ascertain the effect on permeability, or on an active transfer system, of x-irradiated, mature, plant parenchyma tissue by measuring rates of ion uptake and loss. Emphasis was placed on the immediate response in an attempt to distinguish between the physiologic function specifically concerned with salt relations and the various genetic changes which may be harmful to the tissue but are probably not closely related to salt movements.

Materials and Methods

The tissue used in all experiments was that of mature potato tubers (Red Triumph or California Long White). In preparing discs of tissue for uptake or loss tests, a cork borer was used to cut out a cylinder of tissue along the longitudinal axis from the central portion of a potato. This cylinder was subsequently sectioned into thin discs. The dimensions of the discs were 1 mm thick and 22 mm in diameter, except in certain tests as noted below. The discs were washed thoroughly in at least two changes of distilled water before use.

X-irradiation was done using an x-ray tube equipped with a tungsten target and operated at 15 milliamperes at 200 or 250 kvp; no filter was used other than the glass wall of the tube. The dose-rate in various tests was between 900 to 1100 roentgens per minute except as otherwise noted. During exposure, discs, typically, were placed on moist filter paper and covered by cellophane in an open Petri dish. Some treatments of submerged discs and of intact potatoes were at dose-rates as low as 340 roentgens per minute.

Uptake was measured as the rate of accumulation of radioactive rubidium by discs of potato tuber tissue from Rb^{86}-labelled RbCl solution. Counts of fresh moist discs—as selected for uniform weight in the experiments reported here—were found to represent approximately 75 % of the Rb^{86} present as shown by the assay of ashed discs at the end of the tests. Thus the proper correction for self-absorption was made in calculating actual concentrations as in figure 1. Loss of ions from potato discs was measured by the increase in conductance of the external solution.

Some additional details of techniques are given in the descriptions of particular experiments below.

Rubidium Uptake: Previous work has shown that

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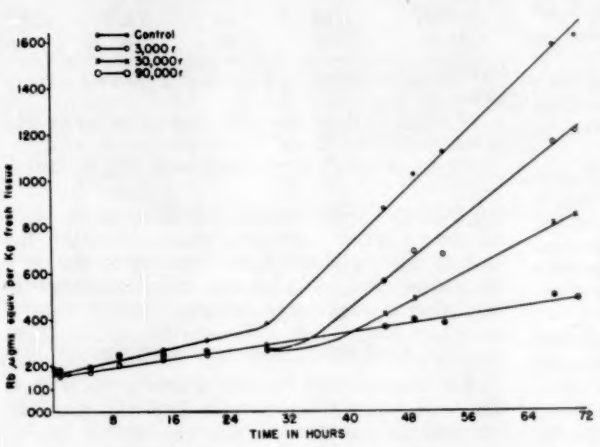
rubidium in appropriate concentrations is not toxic to plant tissue and that it is accumulated by potato discs much as is the case with potassium (6). Therefore, the validity of its use as a test for changes in tissue permeability, or capacity for accumulation, seems adequately established.

In the experiments reported here, potato discs were submerged in vigorously aerated solutions of RbCl, containing radioactive rubidium. The range in activity was 0.01 to 0.1 μc per ml. The solutions were maintained at 22 to 24°C. Uptake by potato discs was followed at different intervals by counts of fresh discs or of ashed sample discs. Ordinarily a single potato supplied sufficient discs for an experiment. Counts for assaying Rb were made using a thin wall or thin window GM tube in a shielded chamber, and a standard scaling circuit.

tarded indicates that the major effect of x-rays is damage to an active absorption system.[4]

The depressing effect of x-rays was observed to increase with dosage in seven different tests. However, in some instances, discs given 3,000 roentgens or less showed a retarded uptake over a period of hours but later accumulated as much Rb as control discs. At 30,000 roentgens, or greater doses, rubidium accumulation varied but was consistently less than in the untreated discs.

Fresh weight was not appreciably affected by x-rays. Control discs and those given 3,000 r or less remained quite turgid; discs receiving 30,000 or 90,000 r frequently became flaccid (in 40 to 100 hours) and had obviously lost in fresh weight. However, some x-irradiation doses greatly retarding Rb uptake had no significant effect on fresh weight.



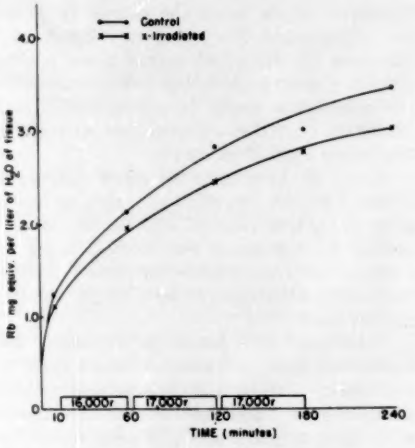


FIG. 1 (*left*). The effect of x-ray dosage on rubidium uptake by discs of potato tuber tissue. Each point represents the average of two discs. External solution 100 μeq RbCl per liter (including Rb^{86} tracer).

FIG. 2 (*right*). The effect of x-rays on rubidium uptake by potato discs during and one hour after x-irradiation. External concentration 10 millimols RbCl per liter.

The nature of the time course of Rb accumulation as found by Steward and Harrison (6) was confirmed. There is a rapid phase of passive uptake during the first hour or two followed by a slower rate of accumulation which may remain relatively constant for days (6). The later phase is known to be related to oxidative respiration and may be referred to as active accumulation. The time course was found to be substantially the same at 1.0, 2.0, and 10.0 milliequivalents RbCl per liter, although greater amounts of Rb were absorbed at the higher concentrations.

The results of the several experiments testing the effects of x-rays on Rb uptake showed variations in response, largely attributed to differences between potatoes. However, within a given experiment, x-irradiated discs showed a conspicuous depression of Rb accumulation over several hours (24 to 60), but no conspicuous immediate effect. A typical experiment is shown in figure 1 in which fresh discs were counted. The fact that the later phase of accumulation is re-

Ting and Zirkle found that x-irradiation at 33,000 r immediately increased the permeability of beef erythrocytes to potassium and sodium (7) and to water (8). Consequently, several uptake experiments were conducted to find the effect of x-irradiation on rate of penetration of Rb into potato discs during the first few hours after irradiation. In figure 2 is shown the result of a test utilizing statistically adequate numbers of discs and taking samples during and following x-irradiation for counts of ashed tissue. In this experiment discs with a diameter of 13 mm and an approximate thickness of 5 mm were used. The experimental solution consisted of 10 millimolar RbCl, containing Rb^{86}, made up in isotonic sucrose solution

[4] The uptake curve here shows an initial lag during the first 24 hours, approximately, in contrast with that of discs which have been prewashed 24 hours; thus the curve in figure 1 differs from those obtained by Steward and Harrison (6). With discs prewashed 24 hours, the present authors found that no initial lag occurred.

(ca 0.24 M) (in order to more closely simulate the conditions of the experiments of Ting and Zirkle). The discs were irradiated in the vigorously aerated experimental solution.

Five samples (a total of 20 discs) were taken at the end of each of the following intervals: 10 minutes and 1, 2, 3, and 4 hours (fig 2). X-irradiation was given at the rate of 340 r per minute, at 15 ma and 200 kvp, during the intervals shown in figure 2.

The final results (fig 2) indicate a slight depression of Rb uptake due to relatively high doses of x-irradiation. Expressing Rb on the basis of tissue water content the average depression for the x-irradiated discs amounted to about 9 % for the three later collections in which the differences were significant at the 5 % level.

The data here indicate that the cells of potato tissue do not respond to x-rays by a marked increase in cation uptake as in the case of erythrocytes (7). In fact, a slight, but apparently significant, decrease occurred. If the initial uptake phase is considered to consist of both passive and active accumulation, then the retardation might be accounted for as the same inhibition of active accumulation as was observed in the longer term experiments.

Loss of Electrolytes from Discs of Potato Tuber Tissue: The effect of x-rays on loss of electrolytes to H_2O, or external solution, by potato discs was studied by measuring the increase in electrical conductance of the external solutions. The discs were prepared and selected from a single potato as in the uptake tests.

Five discs were placed in 200 ml of well-aerated re-distilled H_2O, or nutrient solution at 25°C for each treatment. The increase in conductance of the external medium was measured in each treatment by means of a pair of platinized platinum electrodes (ie, for each beaker) as one arm of a 1,000-cycle per second bridge.

In each test, initial conductance measurements were made on the external solution of two sets of discs previous to any treatment. After establishing the relative uniformity of the two sets of discs with regard to rate of loss of electrolyte, each set of discs was placed in a clean Petri dish with 15 ml of solution during the period of irradiation.

The untreated or control discs were shielded and kept under the same temperature conditions as x-irradiated discs. After irradiation both sets of discs and the solution around them were returned to their original beakers for further measurements. Care was exercised to avoid changes of solution volume and contamination. Differences in conductance developing between the initial reading after x-irradiation and later measurements were the only changes regarded as significant. Electrolyte loss was calculated as KCl per gram fresh tissue per minute.

In the conductance measurements, potato discs showed a steady rate of loss of electrolytes to distilled water from the moment they were immersed. X-ray dosages of 30,000 r and 100,000 r greatly increased the rate of loss of electrolyte to distilled water, but no change was noted at 10,000 r. Results of typical experiments are shown in table I.

The results indicate that rate of loss increases with dosage (see ratio of irradiated to control, S_I/S_C, table I). The variation between potatoes in rate of electrolyte loss (in six experiments) was too great to establish the nature of a dosage response. However, the ratio of the irradiated (S_I) to the control (S_C) at a given dosage invariably showed an increased rate of loss, owing to the x-ray treatments.

Table I

Rate of Loss of Electrolytes by X-irradiated Potato Tuber Discs

Treatment	L * (micromhos / cm^2)	Time (min)	S KCl ** (micro-eq / gm-min)	Ratio † (S_I/S_C)
10,000 r	2.10	51	.370	0.98
Control	2.13	53	.378	
30,000 r	9.50	60	.622	1.25
Control	6.60	52	.498	
100,000 r	7.40	60	.716	2.21
Control	3.50	63	.324	

* Change in specific conductance of external solution (200 ml).

** S KCl = rate of electrolyte loss calculated as KCl in micro-equivalents × gm^{-1} (fresh tissue) × min^{-1}.

† Ratio of S KCl, of irradiated, to S KCl, of control.

Discussion and Conclusions

The results of two types of measurements indicate that with potato tuber tissue x-irradiation depresses rubidium uptake and increases the rate of loss of electrolytes to distilled water. These results are consistent with the idea that x-irradiation damages the mechanisms of salt absorption and retention.

Permeability of potato tissue appeared to be reduced as indicated by the rate of inward movement with the concentration gradient. Actually the slightly slower initial Rb uptake of x-irradiated discs could also be explained as a slowing of an active absorption system rather than a major effect on cellular permeability.

Relatively high dosages of x-rays (3,000 to 100,000 roentgens) gave a relatively small immediate effect on salt relations of mature plant cells as compared to other effects of ionizing radiation. This is in agreement with Hevesy's (2) conclusion regarding work with x-irradiated yeast cells in which a 30,000 roentgen dose gave 90 % lethality (in time), but increased the rate of loss of potassium to the external solution by only 30 %. Thus the mineral relations of potato tuber tissue appear to be relatively insensitive to x-rays in comparison with responses of other tissues, such as elongation of roots or shoots (4), which may be affected at doses as low as 300 roentgens.

Although the effect of x-rays on the mechanisms of tissue regulating ion movement is small in relation to

dose, the response is relatively rapid. Ion uptake, and loss of ions, were measurably altered within an hour. It seems probable that such immediate effects are due primarily to direct alterations of physiologic systems rather than to changes in genes, which then, in turn, result in cellular upsets.

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THE EFFECT OF LIGHT ON THE OXYGEN METABOLISM OF THE PHOTOSYNTHETIC BACTERIUM, RHODOSPIRILLUM RUBRUM[1,2]

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It has been reported that the oxygen consumption of purple bacteria is inhibited by illumination. An account of this phenomenon was given in a review by van Niel (12) where it was interpreted as a respiratory inhibition due to light. This explanation of apparent photoinhibition of respiration in purple bacteria is supported by various kinds of indirect evidence. It is not, however, the only possible interpretation. An alternative was proposed by Nakamura (9) who interpreted the decreased rate of oxygen uptake of illuminated suspensions of purple bacteria as a decrease in net uptake only. Nakamura postulated that the respiratory rate is undiminished in the light and that molecular oxygen actually is produced by bacterial photosynthesis. This production in effect cancels out part of the respiration when the oxygen metabolism is followed manometrically. Van Niel (11, 12) has raised a number of cogent arguments in opposition to Nakamura's interpretation and also to a similar view for a time adhered to by Czurda (4). The chief objections were: (a) the consistent failure to detect oxygen production during bacterial photosynthesis using the extremely sensitive, luminous bacteria method (8) which is, of course, confined to anaerobic conditions, and (b) the absence of any clear demonstration of the alleged oxygen production surpassing the rate of respiration so that net accumulation of the oxygen could be observed.

The present writers have reinvestigated the problem of apparent photoinhibition of respiration, employing a new technique which permits one to distinguish between the above hypotheses. Isotopically enriched oxygen was added to the gas phase in equilibrium with the bacterial suspensions. Using a recording mass spectrometer to monitor, independently, the partial pressures of ordinary oxygen (mass 32) and tracer oxygen (mass 34) it was possible to separate respiration (consumption of both kinds of oxygen) from the production of unlabelled oxygen in the event that any photosynthetic oxygen should appear.

Materials and Methods

Two strains of *Rhodospirillum rubrum* (Esmarch) Molisch were used. Van Niel's strain, S-I, was obtained from Dr. M. D. Kamen, and a strain, designated Athiorhodaceae 1.1, was kindly supplied by Dr. C. B. van Niel. Both strains were maintained in pure culture in 0.2% yeast extract-agar stab cultures. Cultures for experimental purposes were grown on media as described by Gest, Kamen, and Bregoff (6) or by French (5) under anaerobic conditions in four-

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[2] These studies were aided by contract NR 160-030 between the Office of Naval Research and the University of Minnesota. Financial support from the Graduate School and from the Rockefeller Fund is gratefully acknowledged. The isotopically enriched oxygen was prepared by Dr. A. O. C. Nier under a grant from the American Cancer Society through the Committee on Growth of the National Research Council.

[3] Contents of this paper constitute a part of a thesis submitted by J. A. Johnston to the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the Degree of Doctor of Philosophy, while holding an Alexander P. Anderson and Lydia Anderson Fellowship and the Conway MacMillan Memorial Research Fellowship in Botany.

ounce prescription bottles. Rigorous precautions were observed and frequent checks were made to detect any contamination of the cultures by aerobic or anaerobic organisms. The cultures were incubated in a chamber thermostated at 30°C and illuminated by four, 100-watt tungsten bulbs each giving an incident light intensity of 150 fc.

Cells were harvested by centrifugation and washed twice with suspending medium. The suspending medium was either 0.1 M potassium bicarbonate with 0.02 M potassium butyrate as an external hydrogen-donor or 0.1 M phosphate buffer (pH 6.8) with 0.02 M potassium butyrate.

Starved cells were prepared by allowing washed cells, suspended in 50 ml 0.1 M phosphate buffer (pH 6.8) and contained in a cotton stoppered erlenmeyer flask of liter capacity, to stand with occasional shaking for a period of twenty-four hours in the dark at 25°C. Sterile technique was used throughout the preparation of the cells.

All experiments were performed at 21°C. Illumination was supplied by 300-watt, reflector flood lamps, the number being dependent upon the light intensity desired. Light intensity was further controlled by means of a rheostat. Light was directed through a window in the rear of the Warburg bath and reflected into the reaction vessel from below. The relation between incident and effective light intensities was not a simple one because the bacterial content of the experimental suspensions varied from experiment to experiment (20–30 μl packed cells/ml suspension), and the depth of suspension illuminated varied with the design of the particular reaction vessel used. In all instances those light intensities which caused the maximal change in the rate of oxygen uptake were determined and the data reported in this paper pertain to illumination conditions which were sufficient to elicit the maximal change in respiratory rate of the experimental suspensions.

Manometric Method: As has been demonstrated by Burk et al (3) for studies on photosynthesis, it is possible to maintain a constant, high (2–5 %) carbon dioxide pressure by the use of diethanolamine buffers of the Pardee type (10). These buffers can serve as reservoirs, releasing or absorbing carbon dioxide as changes in the carbon dioxide pressure of the gas phase dictates. Under these conditions of constant carbon dioxide pressure, it is possible to measure directly the changes in oxygen pressure regardless of uptake or production of carbon dioxide.

Manometric experiments were carried out using rectangular reaction vessels equipped with two side arms. The diethanolamine buffer was placed in the side arms, and filter paper strips were added to increase the surface area of the buffer. Because the buffer is somewhat slow in coming to equilibrium with gaseous carbon dioxide, all measurements of oxygen pressures reported were made when steady-state conditions prevailed, ie, the transition periods were omitted from the calculations. Sixteen experiments of this type were performed.

Mass Spectrometric Method: Experiments employing tracer oxygen were carried out with a mass spectrometer. A commercial instrument (Consolidated Engineering Co., Model 21-201) was adapted for periodically recording the isotopic composition of the gas phase within the reaction vessel. The manner in which the mass spectrometer was modified for recording a periodic set of analyses was described in detail by Brown et al (2).

The type of reaction vessel and mass spectrometer gas inlet system varied with different experiments. Except for the one described below, the several designs have been described by Brown et al (2, fig 3 and 4), by Mehler and Brown (7), and by Brown (1). In order to provide for use of the same reaction vessel interchangeably with the mass spectrometer and with a conventional Warburg manometer, the system illustrated in figure 1 was devised. The male joint for the vessel was made of stainless steel and housed the mass spectrometer leak as well as inlet and outlet ports

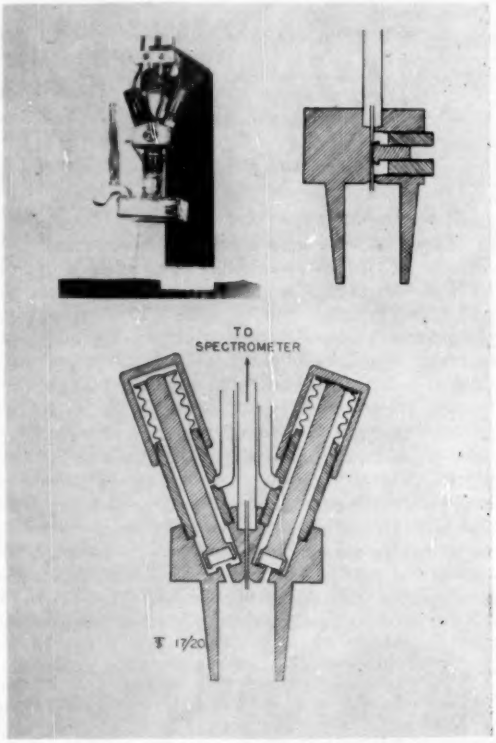

Fig. 1. Mass spectrometer leak and reaction vessel assembly. The leak consists of 0.007 inch (id) copper tubing communicating with the gas space of the reaction vessel and pinched to the required degree of constriction by a plunger driven by a differential screw thread. Flushing ports also enter the vessel within the male joint, and closure is effected by Teflon valve seats. Sylfone bellows allow for travel of the valve stems. One valve is shown open; the other, closed. The assembly is made of stainless steel.

which allowed for flushing the vessel with desired gas mixtures. These openings were closed by valves which were so designed as to provide a minimum of dead space communicating with the main volume of the vessel. With a conventional Warburg vessel in place the whole assembly was mounted on an ordinary manometer holder which oriented the vessel in the constant temperature Warburg bath and provided for shaking the vessel in the usual fashion.

The spectrometer was automatically tuned through a pre-determined sequence of masses so that in most experiments each mass component was analyzed once each minute (in a few instances once each 108 sec). Such analyses originally are in terms of distance of pen travel on a Brown Recording Potentiometer Chart and, with appropriate base line corrections and calibrations, these can be transformed into partial pressures (or, if desired, volumes) of the respective mass components within the gas phase of the reaction vessel. The sequence of such analyses for mass 44 (carbon dioxide), mass 32 (ordinary oxygen), and mass 34 (tracer oxygen) when plotted against time form graphs of the time course of metabolism of each separate component. A graph of this kind for mass 44 has the same significance as has the time course of carbon dioxide metabolism when measured by a conventional manometric method. The data for oxygen (mass 32 and mass 34) would have to be added together to furnish the usual plot of total oxygen metabolism. However we were interested here in the separate processes of oxygen consumption and production (should the latter occur) and one may derive the rates of these separate processes from the data by the following simple computations:

$$U = k[dM_{34}/dt\,(1 + M_{32}/M_{34})] \qquad (1)$$

$$P = k[dM_{32}/dt - dM_{34}/dt\,(M_{32}/M_{34})] \qquad (2)$$

in which: U is the rate of oxygen utilization to be computed, P is the rate of oxygen production to be computed, M_{32} is the observed partial pressure of mass 32, M_{34} is the observed partial pressure of mass 34, and k is a constant the value and dimensions of which depend upon the mass spectrometer calibration, and upon the gas and liquid volumes of the experimental vessel.

It can be seen by inspection of equation (2) that P is equal to zero only when the rates of uptake of both kinds of oxygen are in the same ratio as their partial pressures. In other words, oxygen production could be detected by observing an increase in the prevailing partial pressure ratio, mass 32/mass 34, even though the rate of oxygen consumption were far in excess of the production rate.

Ten experiments were performed with the mass spectrometer.

Results and Discussion

Manometric Experiments: Butyrate respiration in a gas phase containing 5 % carbon dioxide in air was observed manometrically. Table I presents representative data obtained by this method. Two observations, which apply to both manometric and mass spectrometric experiments, may be made: (1) light has a distinct inhibitory effect upon the rate of oxygen uptake and (2) the rate of oxygen uptake in a dark interval following a period of light is less than it is in previous dark intervals. In no instance was complete inhibition of oxygen uptake observed manometrically. The inhibitions in several experiments ranged from 60 to 80 %. Qualitatively our results agree with the results of van Niel and of Nakamura. However, in the experiment cited by van Niel (12), photoinhibition of respiration was complete. In our experiments complete inhibition was observed only once.

The Effect of Oxygen Tension upon the Rate of Oxygen Uptake: At various oxygen tensions, the rate of oxygen uptake during respiration of butyrate in the dark varied in the following way when expressed in arbitrary units:

Gas phase	Rate of O_2 Uptake
O_2, 20 %; CO_2, 5 %; He, 75 %	1.00
O_2, 5 %; CO_2, 5 %; He, 90 %	0.73
O_2, 1 %; CO_2, 5 %; He, 94 %	0.17

It is evident that the rate of oxygen uptake is significantly dependent upon the oxygen content of the gas phase. To rule out the possibility of an apparent inhibition of the rate of oxygen uptake caused by reduced oxygen tension rather than by light, the rate of oxygen uptake was measured during a dark period following each light interval.

Mass Spectrometric Experiments: The effect of light upon butyrate respiration, as measured by using

Table I

Sample Data on the Effects of Light on Oxygen Uptake by *Rhodospirillum rubrum* as Measured Manometrically *

Protocol for vessels #1 and #3	Freshly harvested		Starved	
	1 **	2 **	3 **	4 **
Dark	−3.51	−3.53	−2.74	−2.73
Light	−0.89	−3.52	−0.45	−2.73
Dark	−1.53	−3.50	−2.00	−2.71
Light	...	...	−0.45	−2.70
Dark	...	...	−1.31	−2.65
Cell content ..	47 µl	47 µl	42 µl	42 µl

* Each reaction vessel contained 3.0 ml suspension of cells, grown on the medium of Gest et al, in 0.01 M $KHCO_3$ containing 0.02 M potassium butyrate; each side arm contained 0.5 ml diethanolamine buffer and a filter paper strip. Temperature: 21°C. Gas phase: 5 % CO_2 in air. Incident white light intensity: 200 fc.

** Vessels #1 and #3 were exposed to alternate periods of light and dark while vessels #2 and #4 were kept continuously dark. Each period of light or darkness was of one hour duration but, to avoid transition effects due to lags in absorption or release of carbon dioxide by the buffer, rates were computed from measurements made during the last 30 minutes of each period when a steady state had been achieved. Tabular values are µl O_2 per hour per µl packed cells.

isotopically labelled oxygen, is shown in figures 2A, 2B, and 2C. In the dark and light, oxygen of mass 32 and oxygen of mass 34 were taken up at rates proportional to their partial pressures. The rate of oxygen uptake in the dark was higher than in the light. When compared with the initial rates of oxygen uptake in the dark, the maximal inhibition of respiration by light was 72 %, 84 %, and 79 % as shown in figures 2A, 2B, and 2C respectively. In only one experiment, which is shown in figure 2D, was it possible to obtain essentially complete inhibition of oxygen uptake by light. It should be emphasized that, as was pointed out above, the characteristic inhibitions were not greater than 85 %. Variation in culture medium, in pre-treatment of the organism (freshly harvested or starved or aerobically incubated with substrate), and in suspending medium did not produce an experimental organism which was consistently susceptible to complete inhibition of oxygen uptake by light.

FIG. 2. The effect of light upon respiration of *Rhodospirillum rubrum* in isotopically enriched oxygen-nitrogen mixtures. Light periods are indicated by arrows with the incident light intensity specified. Dark periods are unmarked.

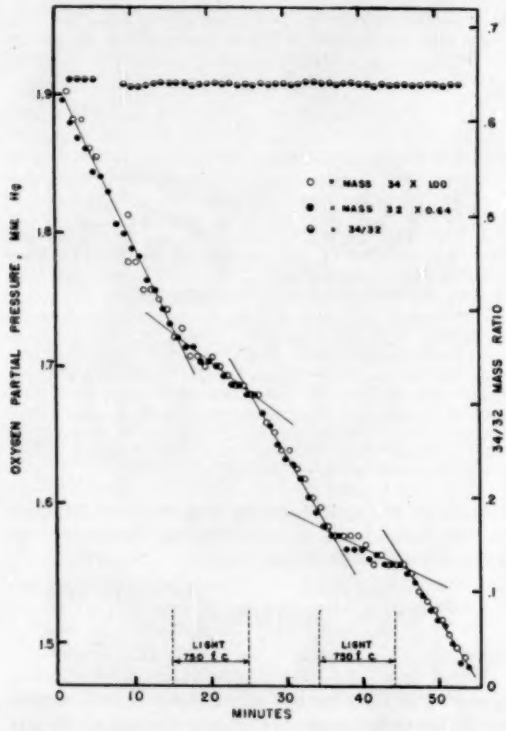

FIG. 2A. The time course of oxygen metabolism of *R. rubrum*. Cells were grown on the medium of Gest et al, starved, and suspended in bicarbonate buffer containing butyrate. The constancy of the isotope ratio, mass 34/32 (half-open circles), indicates that the isotopes were utilized in accordance with their partial pressures. Production of oxygen would have been indicated by a change in this ratio.

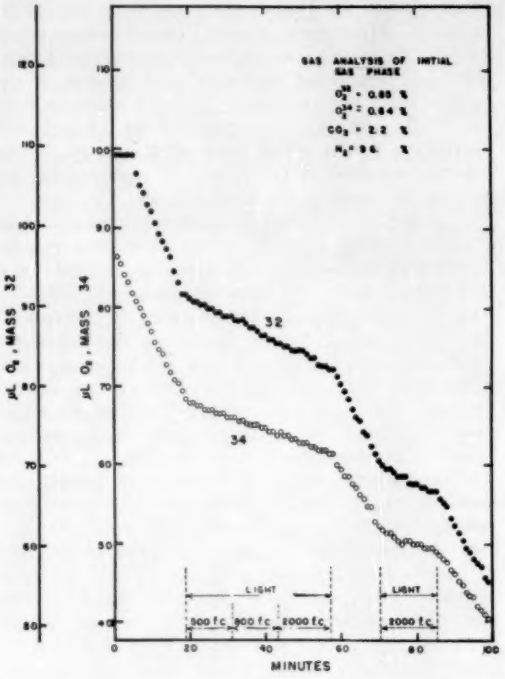

FIG. 2B. The time course of oxygen metabolism of *R. rubrum*. The effect of several incident light intensities upon freshly harvested cells grown on French's medium and suspended in phosphate buffer containing butyrate.

The fate of carbon dioxide is illustrated in figure 2C. In the dark, carbon dioxide was evolved, and at certain light intensities it was taken up. At the higher light intensities carbon dioxide uptake was inhibited. This phenomenon is frequently observed with suspensions of bacteria performing photosynthesis under anaerobic conditions.

In these experiments with tracer oxygen, production of oxygen calculated by equation (2) was in no case significantly greater than zero. Alternatively if we consider the ratio of oxygen, mass 34, to oxygen, mass 32, this ratio remained essentially constant in the light as well as in the dark in all of our experiments. By way of illustration, the ratio is plotted in figure 2A. From the constancy of the 34/32 ratio it must be concluded that *R. rubrum* does not evolve molecular oxygen in the light.

It is relevant to inquire what quantity of oxygen could actually have been produced by these illuminated bacterial suspensions and yet have remained undetected owing to the limitation imposed by the ultimate sensitivity of our measurements. By way of example, in the first light interval of the experiment illustrated by figure 2B, 17 μl oxygen were taken up.

This represents an 84 % inhibition of respiration. By assigning conservative, nominal values to the several errors which attend the application of formula (2) for the calculation of oxygen production, it may be determined that this computation could be in error by 3 μl, an amount of oxygen which, if produced, would have been within experimental error. This value corresponds to only 5 % of the inhibited respiration. Analogous computations on the other experiments lead to similar values. To this extent the precision of the measurements must qualify our conclusion that molecular oxygen production does not occur.

Summary

1. The effect of illumination on the rate of uptake of oxygen isotopes by *Rhodospirillum rubrum* was studied manometrically and also with the use of labelled oxygen in the gas phase. The rate of oxygen consumption was decreased by light and the respiratory photoinhibition varied directly with the light intensity. This phenomenon, previously reported by van Niel on the basis of indirect evidence, now is confirmed by an unequivocal method.

2. It was demonstrated that no molecular oxygen is produced in the light of *R. rubrum*. Therefore, the light effect on respiration is in fact a photoinhibition of oxygen consumption.

3. In general, light did not inhibit completely the oxygen uptake under the conditions studied. Maximal inhibitions observed in most experiments varied from 60 % to 85 %.

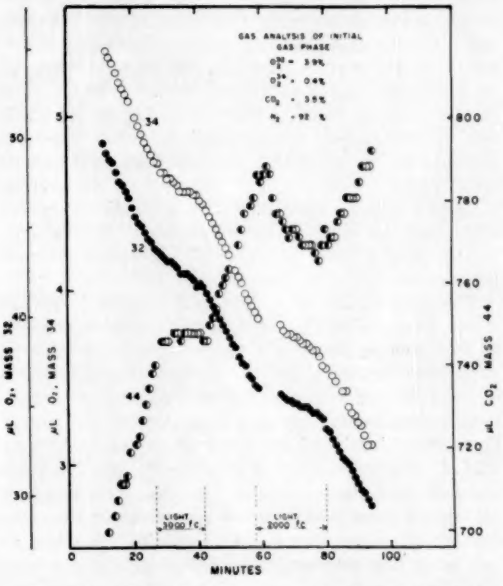

Fig. 2C. The time course of the metabolism of carbon dioxide and of oxygen isotopes by *R. rubrum*. Freshly harvested cells grown on French's medium were suspended in phosphate buffer containing butyrate. At 2,000 fc incident light, bacterial photosynthesis (carbon dioxide uptake) was demonstrated; at 3,000 fc no net change of carbon dioxide pressure was observed.

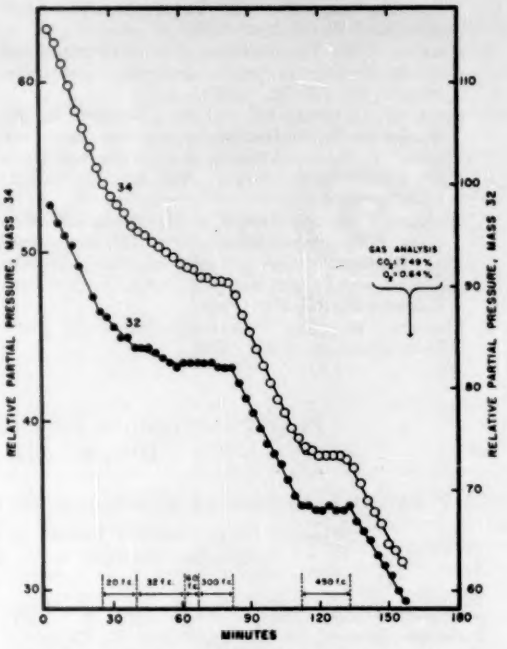

Fig. 2D. The time course of the metabolism of oxygen isotopes by *R. rubrum* in darkness and at various light intensities. Cells were grown on the medium of Gest et al, starved, and suspended in phosphate buffer containing butyrate. Nitrogen was used as the background gas. Essentially complete suppression of the consumption of oxygen isotopes occurred at higher incident light intensities. In this experiment base lines were not obtained. This precludes calculation of absolute ratios of masses 34 and 32.

We wish to express our appreciation to Dr. J. M. Daly and Mr. Arnold Joseph for technical aid and maintenance of the mass spectrometer, to Dr. John H. McClendon for aid in designing the mass spectrometer leak and reaction vessel assembly, and to Miss Wilma Monserude for aid in the preparation of figures and graphs.

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FLUCTUATIONS IN SENSITIVITY OF THE AVENA TEST DUE TO AIR POLLUTANTS [1]

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The Avena test is a biological assay measuring minute quantities of the plant growth hormone auxin, and has been described by Went and Thimann (14). Ever since the test was developed, fluctuations in the sensitivity of the test plants have been noted. Went (11) states that "the maximum angle is different from day to day and fluctuates between 10 and 20° for plants cultivated in water," and further suggests: "I believe that the weather, either sunny or overcast, exerts an effect on the plants even in a darkroom. In view of what we know about the nyctinastic movements of plants it is not excluded that in spite of the uniform conditions in the experimental room, fluctuations in growth have to be attributed to some external factor." These variations in curvature were also mentioned by Maschmann and Laibach (7) in 1933, and were investigated in detail by Kögl, Haagen-Smit and van Hulssen (5). The latter workers found minimum sensitivity to auxin during the day and maximum sensitivity during the late night, but on certain days the rise in sensitivity during night did not occur. Factors causing these fluctuations were not correlated with barometric pressure, cosmic radiation, ionization or other measurable factors; nor was a correlation with sunshine, rain or cloudiness found.

Fluctuations in sensitivity were not found in certain other localities; in Copenhagen, Denmark by Juel (4), in New London, Conn., U.S.A. according to a personal communication from Dr. G. Avery, and in Friday Harbor, Washington, U.S.A. by van Overbeek. Yet, here in Pasadena such fluctuations were observed, and although considerably smaller than in Utrecht, they still are well marked. Went and Thimann (14) have presented a curve showing the phenomenon.

Since the diurnal fluctuation tests at this laboratory were carried out continuously for about 72 hours, several operators generally alternated in carrying them out. To ascertain that the results achieved by different operators were comparable, control tests were run in which the operators worked simultaneously. The results (table I) indicate that variability within single test determinations was greater than between tests carried out by different operators, and that experienced operators (2, 5 and 6) obtained on the average a slightly greater curvature with a smaller standard error than the less experienced workers. With a sufficiently large number of plants all differences between experienced operators disappeared (table II).

The observation of a decreased sensitivity of the Avena plants after the yearly heavy automobile traffic in Pasadena on January 1 suggested exhaust gases as a possible causative agent. To test this possibility, experiments were carried out in two sets of physiological darkrooms, one set receiving its air supply from street level and the other from the laboratory roof about 10 meters above ground. As figures 1A–1B show, diurnal fluctuations in the rooms receiving an air supply from street level were consistently greater than in the other rooms. Fluctuations from test to test were also greater in the rooms with street level air intake (table III).

Experiments were carried out to investigate the effectiveness of an activated carbon filter in removing atmospheric impurities. Air entering one set of rooms was filtered with a Dorex activated carbon filter, but no consistent differences were found in sensitivity between filtered and unfiltered air either with an air

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TABLE I

COMPARISON OF AVENA TESTS CARRIED OUT BY 9 OPERATORS. ALL PLANTS WERE OPERATED ON WITHIN A PERIOD OF 50 MINUTES AND THE GROWING AND RESPONSES ALL OCCURRED IN THE SAME DARKROOM

OPERATOR	CONCENTRATION OF IAA IN μG PER LITER						
	TEST 1			TEST 2			
	25	50	100	0	25	50	100
1. De	6.7 ± 0.57	15.7 ± 1.06	25.9 ± 1.49	0.0	4.1	13.8	21.2
2. Vo	7.5 ± 0.65	18.9 ± 0.87	26.5 ± 1.33	+1.2	4.1	13.0	18.7
3. Po	8.5 ± 0.55	16.9 ± 1.01	26.6 ± 0.90	+0.3	5.0	11.3	16.0
4. Sw	5.7 ± 0.53	17.0 ± 1.09	24.3 ± 1.47	+1.3	4.8	8.8	17.4
5. To	9.1 ± 0.80	18.8 ± 0.94	23.9 ± 1.18	+0.7	8.6	13.4	19.8
6. We	9.2 ± 0.53	19.3 ± 0.63	27.4 ± 1.37	+0.5	3.3	17.7	21.1
7. Wh	8.2 ± 0.65	18.0 ± 0.87	26.5 ± 0.94	+0.8	3.1	13.7	17.9
8. Le	.....	.....	.....	−0.9	6.3	14.4	21.2
9. Be	.....	.....	.....	+0.1	5.5	13.6	16.4
Average	7.8 ± 0.49	17.8 ± 0.49	25.7 ± 0.49	0.4 ± 0.23	5.0 ± 0.57	13.3 ± 0.78	18.9 ± 0.69
Average error of the mean	0.61	0.92	1.24	0.52	0.75	0.84	1.05

intake at street level or roof level (cf fig 1D, 1C). Subsequent experiments showed that the time of contact (about 0.01 second) of the air with the carbon was too short for effective removal.

When attention was focused on air pollutants (smog) causing considerable damage to vegetation in the Los Angeles area (8, 12), it was suggested that these pollutants, showing marked daily and seasonal fluctuation in concentration, might be responsible for the periodicity in sensitivity of the Avena test (12).

MATERIALS AND METHODS

Work herein reported was carried out in the Earhart Plant Research Laboratory (13). One of the rooms used for the Avena tests was supplied air which was passed through a mechanical filter, an electrostatic precipitator, a water spraying system, and four banks of activated charcoal filters—a sufficient number to keep the air in contact with the charcoal about 0.2 seconds. Another room could be supplied with similarly filtered air, with outside unfiltered air, or with filtered air into which a synthetic smog was injected. It had been previously shown by Haagen-Smit et al (2, reviewed by Thomas, 10) that when the gaseous phase of certain olefins was combined with ozone or nitrogen dioxide and sunlight, a series of reaction products was produced which resulted in plant damage identical to that caused by natural smog. This synthetic smog was produced by introducing ozone and a hydrocarbon (gasoline or hexene-1) vapor into a mixing tube, from which it constantly passed into the gas room. The rates were generally adjusted to create an ozone concentration of 0.2 ppm and a hydrocarbon concentration of 5 ppm. before mixing. The hydrocarbon alone caused no plant damage even at concentrations far in excess of 5 ppm. Concentrations were tested in the gas room according to methods in the above paper (2), and ozone was determined by a rubber cracking test (Bradley and Haagen-Smit, 1).

Avena tests were carried out according to the standard methods described by Went and Thimann (14). Two rows of 12 selected plants each were generally used for each treatment, so as to give a sufficient number for statistical analysis. One-mm-thick blocks of 3% agar, which were applied to the plants, were soaked 3 hours in a 0.01 M solution of KCl containing indole-3-acetic acid (IAA) at the concentrations specified. Initial control experiments, in which filtered air was utilized in both rooms simultaneously, demonstrated the necessity of nearly identical cultural con-

TABLE II

COMPARISON OF AVENA TESTS CARRIED OUT BY 3 OPERATORS DURING 3 CONSECUTIVE DAYS (JUNE 7–9, 1940), WITH THE NUMBER OF TEST PLANTS ON WHICH THE AVERAGES ARE BASED BETWEEN PARENTHESES

OPERATOR	IAA CONCENTRATION IN μG/LITER		
	25	50	100
Vo	2.2° (264)	9.4° (264)	16.1° (132)
We	2.9° (308)	9.3° (308)	16.2° (154)
Wh	2.3° (352)	9.4° (352)	16.3° (176)

TABLE III

DIFFERENCE IN CURVATURE IN DEGREES BETWEEN TWO SUCCESSIVE AVENA TESTS WHEN RUN IN TWO DIFFERENT ROOMS, A WITH AIR INTAKE AT STREET LEVEL AND B WITH AIR INTAKE AT ROOF LEVEL (10 m HEIGHT). AVERAGE OF 21 TESTS AT 3 HOUR INTERVALS WITH 22 PLANTS/TEST/IAA CONCENTRATION

ROOM	AIR INTAKE AT	IAA CONCENTRATION IN μG/LITER		
		25	50	100
A	Street level	1.15°	1.76°	4.06°
B	Roof level	0.54°	0.89°	2.00°

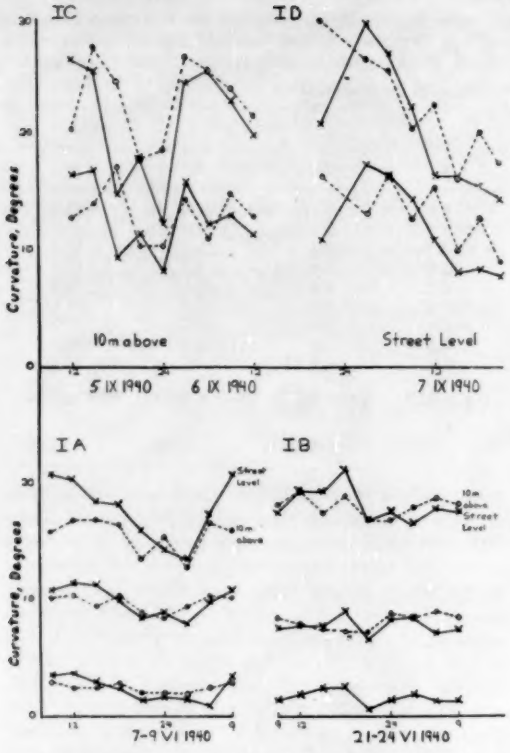

FIG. 1. Fluctuations in sensitivity to IAA in the standard Avena test when carried out at different times of the day. Upper two curves in each figure—concentration of 0.1 mg/liter; next set of two curves—0.05 mg/liter; lowest curves—0.025 mg/liter. Figures IA and IB—averages of tests carried out from June 7–9 and 21–24, 1940. Tests IC and ID carried out with (dotted line) and without (solid line) carbon filter in the air supply for the test run.

ditions to achieve equivalent plant curvatures in the two rooms. The air was maintained at 26.5 ± 0.5°C and 65% relative humidity (92% after agar application). Water level in the germination trays, distance of the plants from the red light, amount of tray left uncovered (3 mm), and placement of the trays in each room so that they received similar amounts of air movement all had to be carefully adjusted. In the experiments which follow, the plants were always removed to filtered air for application of the agar and allowed to so remain until photographed 90 minutes later.

EXPERIMENTAL RESULTS

Once it had been established that synthetic smog given during growth of the Avena coleoptile significantly decreased curvature resulting from subsequent application of auxin, it was considered desirable to find whether or not this decrement in curvature was proportional to the concentration of applied auxin. Experiments were run utilizing varying concentrations of auxin, after the plants had been exposed to low concentrations of synthetic smog. The findings all demonstrated a greater differential in curvature between the plants grown in filtered and in polluted air at higher concentrations of applied auxin. One such experiment is shown in figure 2, in which a very low concentration of synthetic smog (0.1 ppm ozone and 5 ppm gasoline) was administered over a 16 hour period just prior to the second decapitation. IAA was applied in concentrations ranging from 0.04 to 5.0 mg per liter and although curvature was considerably decreased at all concentrations (fig 2), decrement was greater at the higher concentrations. The majority of the following experiments were consequently carried out at a relatively high concentration, giving a so-called maximum angle curvature.

To determine which stages of growth during the 3 day period between soaking of the seeds and photographing the plants were most sensitive to smog, the following experiments were designed. Fumigations were given during the first, second, and third 2 hour periods of the 6 hour interval just prior to agar application, as well as for the entire 6 hours. The agar contained 0.5 mg per liter of IAA. No significant differences in curvature were apparent between any two of the different treatments.

Another experiment was run utilizing 0.5 mg per liter of IAA in which the Avena plants were fumigated during the entire 16 hour period prior to agar application, or during the first or second half of this period. Resultant curvatures are shown in table IV. It is evident that in both 8 hour fumigations, reduction in curvature was just under significance, whereas in the 16 hour fumigation it was significant at the 1% level, accompanied by 16 degrees of freedom.

The effect of synthetic smog given during the 90 minute period of agar application was also studied. In order to maintain a relative humidity of 92% in

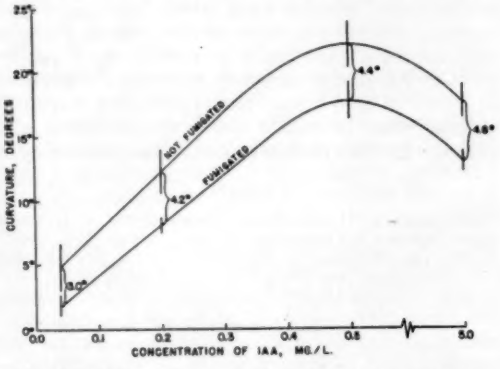

FIG. 2. Reduction of curvature at various IAA concentrations, resulting from the presence of synthetic smog during the 16 hour period prior to agar application. Standard errors of the means, shown as vertical bars, indicate the significant reduction in curvature at higher concentrations of IAA.

TABLE IV

CURVATURE OF AVENA COLEOPTILES WHEN SUBJECTED TO ARTIFICIAL SMOG DURING ALL OR A PART OF THE 16 HOUR PERIOD PRIOR TO AGAR APPLICATION (0.5 MG IAA PER LITER IN ALL CASES)

PORTION OF 16 HOUR PERIOD PRIOR TO AGAR APPLICATION DURING WHICH PLANTS RECEIVED SYNTHETIC SMOG	CURVATURE, DEGREES
Entire period	15.3 ± 0.9
First 8 hours	19.4 ± 1.6
Second 8 hours	17.8 ± 1.2
Control (no smog)	21.9 ± 0.9

the gas room during this period, the volume of air flowing through the room had to be cut to one fifth of its normal rate. The resultant five fold increase in ozone-hydrocarbon concentration over the normal was allowed to remain, since the fumigation period would be comparatively short. Fumigations were carried out during the following portions of the 90 minute period of agar application: (1) entire period, (2) first 45 minutes, (3) last 45 minutes, (4) first 20 minutes, (5) middle 20 minutes, (6) last 20 minutes. Non-fumigated controls were also run in both the filtered air room and the gas room just prior to fumigation. Among the 8 treatments, there was no significant difference in curvature between any two.

To find the effect of smog on resultant curvature when given during earlier stages of germination and development, the growth of the coleoptile was divided into two stages. These stages were termed "germination," which refers to the first two days of germination in the glass trays, and "growth," which refers to the final 24 hour period after the seedlings have been transferred to the glass holders. In the first experiment, plants were fumigated with a low concentration of synthetic smog (0.1 ppm ozone, 5 ppm gasoline) from 9:00 to 17:00 at the end of the germination stage, and 17:00 to 24:00 as well as 8:00 to 17:00 the following day, the end of the growth stage. Plants were fumigated during either one of these stages, both, or not at all. Resultant curvatures (0.16 mg per liter of IAA) are shown in table V.

TABLE V

CURVATURE OF AVENA COLEOPTILES WHEN SUBJECTED TO ARTIFICIAL SMOG DURING VARIOUS PORTIONS OF THE "GERMINATION" STAGE (FIRST 2 DAYS AFTER SOAKING) AND/OR THE "GROWTH" STAGE (THIRD DAY AFTER SOAKING). (0.16 MG IAA PER LITER USED IN EXPT. 1 AND 0.5 IN EXPT. 2)

TYPE OF AIR IN ROOM DURING STAGE SHOWN BELOW		CURVATURE, DEGREES	
GERMINATION	GROWTH	EXPT. 1	EXPT. 2
Smog	Smog	8.7 ± 0.8	18.8 ± 1.5
Smog	Filtered	13.3 ± 1.1	18.3 ± 1.3
Filtered	Smog	20.4 ± 0.8	26.1 ± 2.0
Filtered	Filtered	20.5 ± 1.0	27.5 ± 1.1

A second experiment, of similar design, was also run. In this case, synthetic smog of the same concentration was used, except that hexene-1 was used in place of gasoline. Plants were fumigated during the germination stage from 17:00 to 22:00 the first day, and 8:30 to 14:00 the second day. Fumigations during the final stage were 15:00 to 16:30 and 9:30 to 13:30 the following day, the end of the growth stage. Resultant curvatures (0.5 mg IAA per liter) are also indicated in table V. The data indicate beyond doubt the greater influence of air pollutants when present during the first phases of germination, as compared to a later phase of growth. Also, if one considers the mean of both experiments, curvature was decreased almost as much when smog was given only during the germination period as when it was given during both periods.

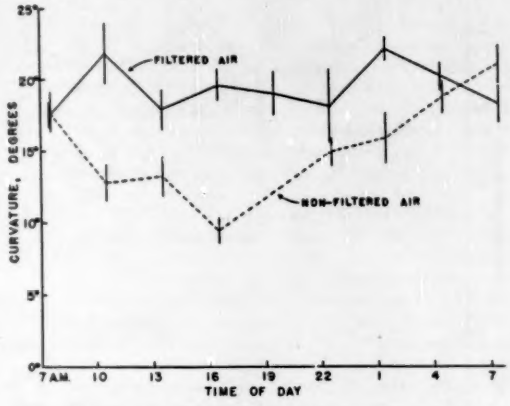

FIG. 3. Diurnal variation in Avena sensitivity to applied auxin as affected by air pollution (dashed curve), and effective removal of pollutants as indicated by absence of diurnal variation (solid curve). The standard errors, plotted for the means, indicate the highly significant (1% level) reduction in curvature at most of the points. Abscissa refers to time of agar application, which ran from 7.30 A.M., Feb. 1 to 7.30 A.M., Feb. 2, 1952.

With some knowledge at hand of the effect of synthetic smog on subsequent hormonal response of the Avena coleoptile, the effect of natural smog was next studied. To find if the previously described diurnal variation of coleoptile sensitivity existed at the present time, and also to determine the efficiency of the filtering system in removing natural air pollutants, the following experiment was carried out. Seeds were germinated every 3 hours over a 24 hour period, half of the seeds being placed each time in the filtered air room, and the other half in the gas room, through which unfiltered outside air was constantly drawn. The experiment was carried out from January 29 through February 2, 1952, a period of light to moderate smog. Transfer to the glass holders, first and second decapitations, and agar application were all carried on at equal intervals after germination, so that the plants were also photographed at 3 hour intervals.

IAA was used at 0.2 mg per liter. As shown in figure 3, a marked maximum in sensitivity appears during the early morning hours and a corresponding minimum during the middle of the afternoon. As to the plants maintained constantly in filtered air, there is no general trend, the variation of individual points apparently being due to the normal variability of biological tests, and of no significance. That the afternoon depression in curvature of plants grown in unfiltered air is highly significant may be clearly seen from the standard errors which are plotted for each point.

To be certain that decrease in coleoptile sensitivity was due to the same air pollutants which reduce visibility and cause eye smarting, another Avena test was run in exactly the same manner, except that it was

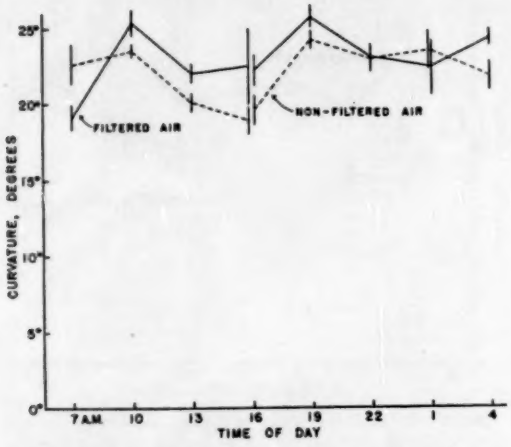

FIG. 4. Lack of diurnal variation in sensitivity to applied auxin when test is carried out during a smog-free period (dashed curve). Response is comparable with that of plants simultaneously grown in filtered air (solid curve). Test started 16:00 March 14 and carried through 16:00 March 15, 1952. The abscissa, however, begins with 7:00 A.M. in order to be comparable with figure 3.

carried out from March 11 through 15, 1952, a period of wind and rain, when no detectible smog was present. IAA was used at a concentration of 0.4 mg per liter. In this case, there was no marked diurnal fluctuation in the plants exposed to non-filtered air, thus indicating that the air was clear of pollutants, and was not further purified by carbon filtration. The resultant curvatures are shown in figure 4.

DISCUSSION

In these investigations it has been shown that the diurnal fluctuations in sensitivity of Avena coleoptiles to auxin are caused by impurities in the air. On the one hand, these impurities can be removed by activated carbon filters. On the other hand, the decrease in sensitivity which is caused by these pollutants can be duplicated by subjecting Avena coleoptiles, especially during their early growth, to artificial smog consisting of peroxides and ozonides of hydrocarbons.

In early work (2) it had been shown that the damage symptoms which are found in the field on leaves of spinach, endive, and other leafy vegetables after periods of smog can be reproduced by subjecting these plants to artificial smog. This smog was produced by mixing ozone and hydrocarbon vapors. Concentrations as low as 0.1 ppm of the resulting ozonides were sufficient to cause damage after a few hours exposure. In this way these sensitive plants could be used to test smog concentrations above the toxic level. However, lower concentrations could not be detected with this biological test. Therefore, it was of interest to try to find a test which would respond quantitatively to very low smog concentrations. It seems that the Avena test answers these requirements.

In the early work on smog it was found that many plants exhibit a decrease in growth rate when they are subjected to smog (6, 3). This decrease in growth could not be overcome by applying auxin to the plants and presumably was not due to a lack of auxin synthesis or to auxin destruction in the plant. This decrease in growth rate in tomato plants was found to occur within 24 hours after fumigation, and after the initial growth retardation, the plants resumed their normal growth rate. Therefore plants which were fumigated for just a few hours soon caught up with the non-fumigated controls and later could hardly be told from the controls. (Insertion on page 203.)

In the work on plant injury (3) it was found that activated carbon filters were the most efficient in removing phytotoxic air pollutants. Other filters in the order of decreasing efficiency were (1) a mixture of iron filings and mossy zinc embedded in steel wool, (2) a mixture consisting of Norite A, peat and sphagnum moss, (3) vermiculite and certain organic and inorganic chemicals, and finally (4) stannous chloride-ferrous sulfate. Filtering agents which were completely ineffective, or nearly so, included cellulose fiber, polyethylene fiber and polyethylene fiber impregnated with motor oil.

The great sensitivity of Avena coleoptiles growing at 26°C and 90% humidity is in line with the sensitivity of tomato and other plants which are most injured at higher temperatures and higher humidities. On the other hand, green plants which are kept in darkness during fumigation are much less sensitive than illuminated plants.

Since exposures to natural smog which do not cause damage to even the most sensitive leafy vegetables are sufficient to cause a highly significant decrease in response in the Avena test, this test may be used as a quantitative assay for very low smog concentrations. A number of older Avena tests show that in earlier days measurable air pollution occurred in Pasadena, which varied also from day to day. The absence of daily fluctuations in sensitivity of the Avena test in Friday Harbor, Washington, and New London, Connecticut, may be an indication of lack of air pollution in those areas.

On the other hand, no visible plant damage was

found to plants in Utrecht, Netherlands, in June and August 1950 nor in October 1952, whereas great fluctuations in Avena sensitivity were described for 1932–1935 (5). And while no fluctuations in Avena sensitivity were found in Copenhagen in the nineteen-thirties (4), fairly extensive smog damage was observed on many plants, including tobacco and conifers, in October 1952.

Summary

A daily and seasonal fluctuation in sensitivity of the Avena test to given concentrations of applied auxin is well known to those who have utilized the test extensively. Usually the greatest sensitivity occurs in the early morning hours. Reasons for this phenomenon have been variously ascribed to magnetic influence of the earth, ionization of the air, variation in the auxin content of the plants, and to air pollutants. That the latter factor is the chief, if not the only factor involved, is supported by this work.

Initial experiments involved fumigation of Avena seedlings with a low concentration of synthetic smog, created by the combination of ozone and hexene-1 or gasoline, and subsequent analysis of the effect by measurement of curvature resulting from applied indole-3-acetic acid. Exposure of the plants to synthetic smog invariably resulted in a decreased curvature, the decrement being directly proportional to the concentration of IAA applied, at concentrations ranging from 0.04 to 5.0 mg per liter. This repressive effect on the plants' response to applied auxin was considerably greater when the fumigation was given during the earlier phases of germination, i.e., during the first and/or second day of germination as compared with the third and final day of growth. In order to cause significant decreases in subsequent curvature, fumigation during the third day (just prior to agar application) had to be very long—approaching 16 hours. Fumigations of various lengths of time given during the 90 minute period of agar application were without effect on curvature.

It was also found that when Avena tests were carried out every three hours over a twenty-four hour period simultaneously in filtered and unfiltered air, the diurnal fluctuation which occurred in the plants grown in unfiltered air was absent in those grown in filtered air, thus indicating that the variability in sensitivity was a result of fluctuating concentrations of air pollutants. A test carried out in the same manner during a smog-free period indicated an absence of diurnal fluctuation even in the case of plants grown in unfiltered air.

It is suggested that the cause of decreased response to applied auxin lies not in the destructive effect of peroxides and other air pollutants upon IAA, but rather in an effect on the growth response of the coleoptile itself.

Also reviewed in the discussion are certain physiological effects of smog on alfalfa, sugar beet, endive, oat, spinach and tomato, and the effect of various environmental factors on the sensitivity of these plants to smog.

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BRIEF PAPERS

A LIGHT ACTIVATED ACCUMULATION OF NIACIN IN TOMATO LEAF DISKS[1,2]

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During the course of an investigation of the pathway of niacin biosynthesis in the leaves of higher plants, evidence has been obtained that light has a role in the process. This paper reports some experiments showing that niacin accumulates in tomato leaf disks when they are exposed to light.

Leaf disks were cut from healthy, fully expanded leaves taken from mature tomato plants grown in the greenhouse. Samples of twenty disks each were selected at random and placed in water during exposure to the experimental conditions. For determination of the initial niacin content, equal samples of disks were frozen immediately at −20°C. This freezing has been found (4) to protect the niacin content of plant tissue for several weeks. The samples exposed to experimental conditions were likewise frozen after a specific incubation time.

The fresh weight and dry weight were determined with separate samples of disks. Since preliminary experiments showed no significant change in weight occurred during short incubation periods (six hours or less), weight determinations were made only at the start of the experiment. For longer incubation periods weights were determined both before and after incubation.

To guard against possible bacterial contamination, all disks to be incubated for more than six hours were surface sterilized by soaking in 0.5 % sodium hypochlorite for ten minutes before incubation.

The total niacin content of the disks was determined by the microbiological method (1) using *Lactobacillus arabinosus* 17-5.

Recovery of niacin was determined by assay of two equal samples of disks, to one of which a known amount of niacin had been added. Recoveries of 105, 99, and 94 % were obtained in three experiments.

The significance of results was determined by comparison of the results obtained in a series of separate experiments. A repeated change in niacin content of 5 μg per gm dry weight, or about 10 % of the initial niacin content, has been taken as a significant change. This is based on the fact that in 26 replicate samples from the first 50 experiments the difference in niacin content averaged 1.5 μg per gm dry weight, with a maximum difference of 6.1 and a minimum difference of 0.0 μg per gm dry weight. In four experiments in which samples were incubated in triplicate the largest standard error was ± 1.2 μg per gm dry weight.

The results of some representative experiments showing the accumulation of niacin in leaf disks exposed to light are presented in table I. The light intensity at the base of the flasks containing the leaf disks was 50 fc, with illumination provided by a 100-watt tungsten bulb placed under a water bath, equidistant from all samples. Other flasks were kept in darkness. The temperature difference between light and dark treatments was always less than one degree C. Since preliminary experiments showed that vacuum infiltration of the disks with water did not affect the reaction, the disks were infiltrated to make

TABLE I

NIACIN CONTENT OF LEAF DISKS BEFORE AND AFTER INCUBATION IN LIGHT AND DARK

EXPERIMENT	INCUBATION CONDITIONS		NIACIN CONTENT	
	Time hrs	*Exposure*	*μg/gm dry weight*	*μg/disk*
68	0	.....	46.6	0.128
	3.5	Light	56.3	0.155
	3.5	Dark	44.1	0.121
69	0	.....	38.9	0.117
	3	Light	44.3	0.133
	3	Dark	39.0	0.117
73	0	.....	50.6	0.127
	4	Light	58.2	0.146
	4	Dark	49.6	0.124
76	0	.....	54.8	0.154
	3.5	Light	64.3	0.180
	3.5	Dark	55.5	0.156
139	0	.....	38.4	0.112
	18	Light	70.0	0.203
151	0	.....	44.6	0.169
	24	Light	87.3	0.289
	24	Dark	48.9	0.162

TABLE II

EFFECT OF LIGHT INTENSITY ON THE ACCUMULATION OF NIACIN IN LEAF DISKS INCUBATED AT 24°C *

LIGHT INTENSITY	NIACIN INCREASE	
	3 HR INCUBATION	5 HR INCUBATION
foot-candles	*μg/gm dry wt*	
0	0.3	1.1
10	12.1	13.6
25	17.4	22.6
75	17.5	25.3
200	17.4	24.1

* Initial niacin content 48.9 μg/gm dry weight.

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[2] Paper from the Department of Botany, University of Michigan, no. 998.

them translucent and to help provide even distribution of light.

A comparison of the niacin content of the incubated disks with that of the unincubated clearly shows that the niacin content increases in light either on a per disk or dry weight basis. There is no significant change in niacin content in the dark on either basis. These observations have been amply verified by the results of more than 50 experiments in which similar conditions prevailed.

The effect of light intensity on the rate of niacin increase was studied by suspending flasks with samples of leaf disks at increasing heights above two 100-watt bulbs, in positions where the light intensity had previously been determined with a model DW, G.E. light meter. The disks at zero intensity were in a light-tight container beside the incubation apparatus. A water bath was placed over the bulbs to prevent temperature changes in the samples. The results of this experiment are shown in table II.

The fact that the niacin increase during the first three hours is the same at all light intensities above 25 fc indicates that the reaction has become light saturated at about that intensity. A light saturation at such a low light intensity is not generally characteristic of the process of photosynthesis (6), but exchange of gases might limit the process in this experiment.

Another experiment of this type was run, with the same results. It has also been found that leaf disks exposed to full sunlight do not increase in niacin content any faster than disks exposed to 50 fc.

To determine if the light activation was caused by photosynthetic production of sugar, samples of disks were incubated for four hours in light and dark after infiltration with either 1 or 2% sucrose solutions. The sucrose did not cause niacin accumulation in dark, nor did it increase the amount formed in light.

The heat lability of the system was determined by boiling samples of disks for 2 to 3 minutes in flasks and then incubating them in light for periods of from 3 to 16 hours. The niacin content of the boiled samples after incubation was compared to the niacin content of samples incubated without pretreatment, and of samples frozen without incubation. The niacin content of the boiled disks did not change during incubation, which indicates that the system responsible for accumulation is thermolabile.

TABLE III

EFFECT OF TEMPERATURE ON THE ACCUMULATION OF NIACIN IN LEAF DISKS INCUBATED IN LIGHT*

INCUBATION TIME	NIACIN INCREASE	
	26°C	37°C
hrs	*μg/gm dry wt*	
3	4.4	10.5
10	10.5	15.9
22	18.8	...

* Initial niacin content 33.0 μg/gm dry weight.

To investigate the effect of temperature on reaction rate, samples of disks were incubated in water baths at 26° and 37°C at a light intensity of approximately 75 fc. The results of one experiment are presented in table III.

At both temperatures the rate of niacin accumulation tends to decrease after several hours incubation. This rate decrease has been observed in other experiments. The Q_{10} of the reaction over this temperature range is 2.2 with an incubation time of 3 hours. In another experiment the Q_{10} was 2.0 over the same temperature range with an incubation time of 4 hours. The effect of temperature on the reaction indicates that thermochemical reactions are involved, presumably enzyme catalyzed.

As further experiments were run, it was found that within any group of tomato plants of the same age the niacin content of the leaves increased during the morning, and decreased during the afternoon and evening. The increase of niacin in leaf disks, however, was found to occur regardless of the time of cutting, and was not limited to periods when an increase of niacin normally occurs in leaves attached to the plant. Thus the synthesis of niacin in leaf disks would not be primarily a function of the normal diurnal variation.

The evidence that the niacin increase in leaf disks was not an expression of a similar increase in leaves attached to the plant led to an investigation of the wounding effect caused by cutting the leaf disks. It is well known that wounding plant tissue causes an increase in respiration (7); and a hormone which stimulates cell division has been isolated from wounded tissue (3). LaRue (5) has shown that wound hormones which stimulate the rooting of cuttings can be extracted from tomato leaves.

To evaluate the effect of wounding, the increase of niacin in intact detached leaflets was studied. Six pairs of leaflets from the upper leaves of tomato plants were cut and weighed. By this method the wounding caused by disk cutting is largely eliminated. Six of the leaflets were incubated in light for 3.5 hours, while the other six members of the matched pairs were frozen immediately. In two separate experiments the niacin content of the incubated leaflets was 22 and 35% higher than that of the unincubated. The increase of niacin content in these leaflets is taken as an indication that wounding during disk cutting is not the cause of niacin synthesis in leaf disks, but that the accumulation observed is initiated by detaching the leaf. Detached leaflets incubated in the dark did not increase in niacin content.

As a further check on wounding effect, disks were washed more than usual before incubation to see if the niacin increase was reduced. Several experiments were run in which disks were washed with running tap water, then soaked in tap water at 5°C for as long as three days before they were incubated. In every instance the synthesis in the washed disks was not significantly different from that in disks from the same sampling incubated immediately after cutting. The fact that synthesis was not decreased serves to

support the conclusion above that niacin increase is not initiated by wounding.

It is emphasized that the increase of niacin in leaf disks must be considered as a net result of synthesis minus destruction during the incubation period. It is clear from the results presented that a rapid synthesis of niacin occurs in tomato leaf disks incubated in light. Whether the actual rate of synthesis is lowered by concurrent destruction cannot be determined, but experience with leaf disks in the dark indicates that niacin destruction, in darkness at least, is a rather slow process.

The synthesis of niacin in the light is apparently not related to the photosynthetic production of sugar, since the light intensity at which the reaction reaches its maximum rate is too low to cause appreciable photosynthesis in the leaf disks. This is further indicated by the fact that addition of sucrose will not cause synthesis in the dark. A study of the action spectrum of the reaction, among other things, would be desirable to eliminate photosynthesis completely from consideration.

The magnitude of the reaction, in some cases resulting in a doubling of the niacin content of the leaf disks in 18 hours, is sufficient to be considered important in the niacin economy of the plant. Since the synthesis is apparently not a result of wounding during disk cutting, the most obvious suggestion is that the niacin accumulates because the normal translocation to other parts of the plant is interrupted. The fact that the roots of many plants, including certain varieties of tomato (2), are dependent on an external source of niacin for continued growth when excised from the plant may be taken as an indication that the leaves could be the natural source of the vitamin for the rest of the plant. Furthermore, the increased niacin content of tomato plants under long photoperiods recently reported by Gustafson (4), as well as the increase of niacin during the morning in leaves attached to the plant, observed in conjunction with the present experiments, may be a manifestation of a light activated niacin producing system in normal operation.

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B VITAMINS IN STARCHY AND SUGARY MAIZE ENDOSPERMS[1, 2]

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Study of the interrelationship between genes and metabolism has provided many examples of biochemical reactions under hereditary control. In some cases it has been possible to assign a fairly simple role to the chemical result of gene action, for instance where a single chemical difference predominates as in many growth-requiring mutants of *Neurospora*, or as in flower color mutants where a difference in structure of the pigment molecule may be the sole obvious chemical change associated with a gene change (6).

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[2] This work was supported by funds from the Atomic Energy Commission administered through contract with the Office of Naval Research, United States Navy (Contract N6-onr-244, Task Order 5, Project NR-164-340).

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In some instances, as in the starchy-sugary alleles, a gene difference can condition such a series of effects that analysis of the primary gene action becomes difficult. Quantitative chemical differences between starchy (Su_1) and sugary (su_1) maize have been reported for a variety of substances. Sugary kernels or endosperms have a higher content of: protein, fat (16), water soluble polysaccharides, reducing sugars, sucrose (10), tryptophan (28), indoleacetic acid (2), and niacin (3). Recent attempts to define the action of the starchy-sugary alleles have been predicated on the hypothesis that one gene-caused biochemical difference gives rise to the whole biochemical and morphological expression of the character by secondary reactions; that is, a single chemical effect interacting with a variety of systems gives rise to the various pleiotropic effects of the sugary gene. The problem

of gene-action-chemistry consists of tracing these interactions to the primary cause. Investigation of the starchy and sugary alleles has thus centered about (a) finding the first characteristic chemical differences to appear in the course of kernel ontogeny (27), and (b) assaying kernels for substances that are known or suspected of being metabolically interrelated (28). The present study has been directed toward finding how B vitamin concentrations are influenced by the starchy-sugary alleles. The role of B vitamins as catalytic factors in cellular metabolism suggested that at some level one or more B vitamins might be causally related in the differentiation of the starchy and sugary phenotypes.

Materials and Methods

The maize samples used for this study were of four types: (a) ears segregating starchy and sugary, with genetic backgrounds that include T 13, P 51, US 34, and Zapaluta chica (Mexican); (b) three ears, each segregating for a different radiation-induced new occurrence of sugary; (c) converted sugary lines in which sugary has been repeatedly crossed to and reisolated from a standard inbred line (in this case converted sugary and inbred lines are compared), and (d) segregating ears from selfed heterozygous sugary conversions. The background of (a) and (b) types of samples were heterogeneous, those of (c) and (d) types were fairly uniform, since, by backcrossing, the starchy and sugary lines tended to become isogenic except for the starchy and sugary genes.

Kernels for assay were degermed and ground to 40 mesh. The material termed "endosperm" thus consisted of true endosperm plus the thin pericarp layer. Samples were protected from light and kept tightly stoppered in the refrigerator. All determinations were carried out by published microbiological methods. Assays are reported on the basis of air-dried kernels.

B_6 was determined by the use of *Neurospora sitophila* mutant 299 (25). Samples were hydrolyzed with 4 *N* sulfuric acid by autoclaving 2 hours (25). After inoculation the flasks were incubated at 25°C for 72 hours and growth measured as dry weight of mycelial pads. Since thiamine stimulates this mutant, an excess was added to the basal medium as recommended by Tatum et al (25). This procedure obviates the necessity of destroying thiamine in the samples and increases the sensitivity of the assay. Mutant 299 gives approximately equal molar response to pyridoxine, pyridoxal, and pyridoxamine (22), thus the values reported are for total B_6, calculated as pyridoxine.

Biotin was determined by the use of *Neurospora crassa* wild-type strain number 4. Samples were hydrolyzed and incubated as for B_6.

Inositol was assayed by the procedure of Beadle (5) using *N. crassa* mutant number 37401. Sample treatment and incubation were the same as for B_6.

Choline was determined by the use of *N. crassa* mutant number 34486 by the method of Horowitz and Beadle (13). Samples were hydrolyzed and cultures incubated as for B_6. Adsorption on permutit columns was deemed unnecessary since the low, approximately 0.1 to 0.2 percent, concentration of methionine in ground maize (4) should not interfere with choline determination. This assumption was verified by comparing standard choline response curves without methionine and with methionine at twice the level expected in the samples. This amount of methionine supplement caused only a slight difference (about two percent stimulation). The higher protein content of su_1 endosperm was calculated to be of insufficient magnitude to affect the choline determinations through methionine stimulation.

Pantothenic acid was determined by turbidimetric estimation of growth of *Lactobacillus arabinosus* after 18 hours (21). Samples were hydrolyzed by autoclaving with distilled water for 15 minutes.

Riboflavin was determined by titration of the acid produced by *Lactobacillus casei* at the end of 72 hours (23). Samples were hydrolyzed by autoclaving with 0.1 *N* hydrochloric acid for 15 minutes. Riboflavin solutions and samples for assay were protected from light as much as possible during storage and handling.

Thiamine was assayed by turbidimetric measurement of the growth of *Lactobacillus fermentum* after 16 hours incubation (19). Samples were hydrolyzed by steaming with 0.1 *N* sulfuric acid for 30 minutes, followed by simultaneous taka diastase and papain digestion at pH 4.5.

Niacin was assayed by titration of the acid produced by *Lactobacillus arabinosus* after 72 hours growth (1). Samples were hydrolyzed by autoclaving with 1 *N* sulfuric acid for 30 minutes.

Lactobacillus casei and *L. fermentum* were tested for the reported growth stimulation by certain carbohydrates (11). *L. fermentum* gave appreciably higher values when the basal medium containing suboptimal amounts of vitamins was supplemented with maltose; therefore, an excess of this sugar was added to the basal medium for thiamine assay to eliminate possible differential stimulating effects of starchy and sugary material because of carbohydrate differences.

Results and Discussion

The data for eight B vitamins are presented in table I on a weight basis and summarized in table II on a weight and kernel basis. All eight vitamins are significantly higher in sugary on a weight basis, while inositol, biotin, pantothenic acid, niacin, and thiamine are significantly higher on a per endosperm basis as well. The presence of higher thiamine and biotin levels in endosperms of $su_1\ su_1\ su_1$ versus $su_1^{am}\ su_1^{am}\ su_1^{am}$ (genotypes 3-3 and 0-3) had earlier suggested that the relationship of sugary and niacin might not be specific for this vitamin (7). The present data clearly indicate the general nature of the vitamin differences and point to the need for a general explanation of higher B vitamin concentrations.

The vitamin levels reported here are in reasonable agreement with ones from literature, especially in view of differences in the methods employed, the relatively few assay values available in some cases, and in par-

TABLE I

B VITAMIN CONTENT OF STARCHY AND SUGARY MAIZE ENDOSPERM *

SOURCE **	KERNEL TYPE	VITAMIN CONTENT, μG PER GRAM							
		RIBOFLAV.	INOSITOL	CHOLINE	B_6	BIOTIN	PANT. ACID	NIACIN	THIAMINE
a	*Su*	0.92	210	552	3.48	0.067	1.92	24.5	0.218
	su	1.37	402	804	4.57	0.149	8.70	41.0	0.736
a	*Su*	1.39	186	647	4.07	0.069	2.50	18.1	0.448
	su	1.51	216	706	4.93	0.089	4.83	25.4	1.090
a	*Su*	1.52	142	426	2.68	0.063	2.54	14.4	0.600
	su	2.22	399	680	4.55	0.084	7.55	46.5	1.470
a	*Su*	1.27	163	483	4.11	0.068	1.77	28.4	0.190
	su	1.45	177	500	5.58	0.075	2.69	46.8	0.242
b	*Su*	1.15	133	655	2.31	0.054	1.89	17.8	0.491
	su	1.22	155	878	4.32	0.096	4.63	32.0	0.789
b	*Su*	1.04	100	528	2.56	0.039	1.48	20.3	0.364
	su	2.11	145	623	2.86	0.078	5.94	42.8	0.752
b	*Su*	1.06	92	714	2.93	0.051	2.00	8.9	0.282
	su	1.05	142	817	4.95	0.081	2.94	15.2	0.507
c	*Su*	1.02	147	603	2.43	0.048	2.30	39.8	0.265
	su	1.76	420	826	4.93	0.102	4.87	66.3	1.890
c	*Su*	1.79	187	473	3.57	0.075	4.53	36.4	1.170
	su	1.77	247	545	4.13	0.099	10.73	82.5	2.130
c	*Su*	1.60	337	484	4.28	0.063	2.44	27.4	0.389
	su	1.73	355	784	5.67	0.104	3.87	55.2	2.710
d	*Su*	1.78	161	490	4.83	0.055	1.89	21.0	0.545
	su	1.65	330	615	6.02	0.095	6.14	64.7	2.370
d	*Su*	1.29	74	308	3.50	0.043	2.60	24.1	0.382
	su	2.26	174	564	5.36	0.071	7.00	60.9	0.833

* Endosperm + pericarp.
** See text, a = Miscellaneous segregating ears.
b = Segregating new occurrences of *su*.
c = Inbred lines vs *su* conversions.
d = Segregating ears from heterozygous conversions.

ticular the attempt in this study to obtain a diverse series of samples with the anticipated range of values. Comparisons of thiamine content should be made cautiously, since tissue constitution of the sample is very important. Maize germ or embryo contains about 18–20 μg of the vitamin per gm whereas the endosperm contains only 1 to 2 μg per gm (9). Thiamine levels reported here are in good agreement with values for maize samples described as lacking germ (7, 9), but are considerably lower than levels reported for whole grain (14, 20). Release of thiamine bound as cocarboxylase has been assured by the enzymatic digestion employed (8). Biocytin, the lysine peptide form of biotin, is available to *Neurospora* (29) and so if present is included in biotin determinations. Pantothenic acid levels reported here represent free or very readily available forms of the vitamin and do not include pantothenic acid bound as coenzyme A. Comparison of literature values for wheat, hydrolyzed by means of distilled water, with values obtained following special enzymatic treatment to release coenzyme A–bound pantothenic acid (17) suggests that, in this grain at least, the major portion of the pantothenic acid is free or easily eluted.

The use of "endosperm" that includes the thin adhering maternal pericarp tissue should not have introduced a large error since: (a) pericarp makes up only ca 6 to 9 percent of the "endosperm" (26), (b)

TABLE II

SUMMARY OF B VITAMIN LEVELS ON PER GRAM AND PER ENDOSPERM * BASIS

BASIS	KERNEL PHENOTYPE	AVERAGE VITAMIN CONTENT IN μG							
		RIBOFLAV.	INOSITOL	CHOLINE	B_6	BIOTIN	PANT. ACID	NIACIN	THIAMINE
Per gm	*Su*	1.32	161	530	3.396	0.0579	2.32	23.3	0.445
	su	1.68	263	695	4.822	0.0936	5.82	48.4	1.293
Per endosperm	*Su*	0.266	31.7	109	0.682	0.0118	0.452	4.49	0.086
	su	0.254	40.1	105	0.730	0.0142	0.860	6.94	0.184
Per gm	P ** =	0.02	< 0.01	< 0.01	< 0.01	< 0.01 (ca 0.03)	< 0.01	< 0.01	< 0.01
Per endosperm	P ** =	> 0.4	0.05	0.5	0.3	< 0.05	< 0.01	< 0.01	< 0.01

* Endosperm + pericarp.
** Probability that observed differences are due to chance; obtained by the t test using paired values.

most samples are from segregating ears and the pericarp tissue being maternal in origin has the same constitution for starchy and sugary kernels, and (c) the pericarp may have a low content of all vitamins as suggested by low levels found for niacin in maize (12), and other B vitamins in wheat (24).

In the case of a gene, such as sugary, which is reported to have many diverse effects, there arises the question of whether all the differences are due to the sugary gene, or whether some of the effects may be traceable to a closely linked gene or genes fortuitously present in the sugary stocks that have been assayed. From a consideration of their own and other data showing a consistent association of sugary phenotype and higher niacin level, Leng et al (15) have concluded that linked genes are not involved in the determination of niacin concentration in sugary. However, sugary lines that have been tested might have shown morphological and biochemical characteristics partly because of the sugary gene and partly because of genes very closely linked to sugary that have been transmitted as a block from some ancestor common to all or most sugary lines. It is unlikely that separate occurrences of sugary mutations would also carry mutated genes for specific closely linked loci. It is extremely unlikely that three new occurrences of sugary would all be mutated for such special loci. Thus it is almost certain that the separate occurrences of sugary will represent mutations at the sugary locus only. We have, therefore, two simple possibilities for the vitamin levels of new occurrences of sugary: a) if the sugary locus alone is responsible for the effects attributed to it, then the new sugary lines will resemble other sugary lines in their influence on vitamin levels, or b) if the sugary locus is responsible for only some of the differences that have been reported, as for instance for the carbohydrate concentration, and other genes closely linked to it determine other differences, such as the vitamin levels, then the new sugaries might be expected to resemble starchy with respect to some or all of their vitamin levels. It can be seen in table I that the three new occurrences of sugary (source b samples) show vitamin level differences similar to the ordinary sugary types. This is strong evidence that the sugary gene itself is responsible for the differences in vitamin level.

The present B vitamin determinations have added four more substances on a per kernel basis or seven more on a weight basis that are higher in sugary than starchy maize. Taken with the chemical differences that have already been reported, the starchy-sugary alleles are seen to be pleiotropic indeed. No specific mechanism underlying the relationship between carbohydrates and B vitamins can be suggested, although the comparison of starchy versus sugary endosperm, and vegetative versus storage tissue, with respect to metabolic level (18) is interesting, since such a broad basis is needed to encompass the carbohydrate and vitamin differences now known. The recent finding that approximately 90 percent of the extra niacin of sugary endosperm is localized in the aleurone layer, which is thicker in sugary than starchy (26), may provide a more specific basis for higher vitamin levels in sugary. In this way a "morphological" feature of sugary, ie, thicker aleurone, can be envisaged as having a biochemical consequence. Thus, if inositol, biotin, pantothenic acid, and thiamine are, like niacin, sufficiently concentrated in the aleurone, the greater amount of aleurone in sugary may also account for these vitamins being higher in sugary endosperm. On the other hand, riboflavin, choline, and B_6 might be higher only on a weight basis because of being less differentially distributed between aleurone and inner endosperm or because of being less concentrated in sugary than starchy aleurone. In future analyses it will be necessary to consider aleurone and inner endosperm separately, in order to take into account the specialization of aleurone tissue as evidenced by the expression of certain color genes and differences in the concentration of protein and niacin.

Summary

Twelve paired samples of sugary and starchy maize endosperm from segregating ears or closely related lines were assayed for choline, B_6, riboflavin, inositol, niacin, biotin, pantothenic acid, and thiamine by microbiological methods. All eight B vitamins were significantly higher in sugary than starchy on a percent basis, but only the last five were higher on a per endosperm basis. Assays on new radiation-induced occurrences of sugary indicate that the differences found are a function of the sugary gene and not other even closely linked genes.

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THICKER ALEURONE INADEQUATE EXPLANATION FOR HIGHER NIACIN IN SUGARY MAIZE[1, 2]

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A recent paper by Teas (4) reports the niacin in sugary and starchy kernels from two segregating ears of corn and draws certain conclusions which appear questionable. Teas concludes that "the higher niacin has a morphological basis in that sugary kernels have more niacin-rich aleurone tissue than starchy." He proceeds: "This finding makes suggested biochemical explanations of the carbohydrate-niacin differences unnecesary." His citations at that point are to our citations (1, 3). In both of these papers possible physiologic explanations are suggested for the higher niacin *concentration* in sugary segregates. Teas et al (5) show graphically the course of niacin content in sweet and starchy kernels during development. Their graphs are based on absolute quantities per kernel rather than concentrations. They also discuss possible physiologic explanations for the niacin differences. It is such suggested explanations (1, 3, 5) that Teas (4) states are made unnecessary by his current observations.

It is desired first to consider briefly the propriety of applying results on a per-kernel basis to an interpretation of a phenomenon based on concentration. It is of course true, as stated by Teas et al (5), that a per kernel basis may avoid large percentage changes

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in some substances caused by the rapid accumulation of reserve carbohydrates as kernels mature. The niacin problem, however, concerns the niacin in sugary and starchy kernels at maturity, and its possible relation to the nature of the reserve carbohydrates may be of the essence. Furthermore, quantity per kernel also may be misleading. For example, Richey and Dawson (3) report the niacin in normal (*De*) and defective (*de*) kernels on several segregating ears. The only visible effect of *de* on these kernels was their smaller size. There was no statistically significant difference in niacin concentration between the *De* and *de* kernels although the latter contained only 60 % as much niacin per kernel. Thus, the two kinds of kernels, with apparently equal opportunity, accumulated niacin in the same relation to kernel weight, largely starch, but not equally per kernel.

increase of niacin is not due to the embryos." But, for ear 1, the sugary embryos contained 0.728 μg of niacin compared with 0.498 for the starchy embryos. Accordingly, one might also conclude the excess is not due to the endosperms, inasmuch as whole and deendospermed kernels both show higher niacin.

The last three columns of table I were calculated from the appropriate preceding columns. The contributions of every part in μg per gm of the whole kernel also are larger for the sugary kernels. Moreover, the ratios for these concentration contributions in the last column of the table agree in ranking with the ratios for absolute contributions in the third preceding column. The concentration ratios are consistently larger, a reflection of the fact that the sugary kernels were only about two thirds as heavy as the starchy (Ear 1). It is the excess of niacin concentration in

TABLE I

NIACIN AND NIACIN CONCENTRATION IN DISSECTED FRACTIONS OF STARCHY AND SUGARY KERNELS FROM TWO SEGREGATING EARS OF CORN

KERNEL PART	DISTRIBUTION BY WEIGHT		μG OF NIACIN							
			IN KERNEL PARTS					PER GM OF KERNEL		
	%		PER GM		PER PART					
	Su	*su*	*Su*	*su*	*Su*	*su*	*su/Su*	*Su*	*su*	*su/Su*
	*	*	*	*	*	*	**	**	**	**
Ear 1										
Pericarp	5.4	8.0	7.8	7.8	0.103	0.111	1.08	0.42	0.62	1.48
Aleurone	2.2	6.0	545.0	372.0	2.940	3.982	1.35	12.00	22.32	1.86
Inner endosperm	81.6	68.8	3.2	6.3	.647	.779	1.20	2.61	4.33	1.66
Embryo	10.8	17.2	18.8	23.7	.498	.728	1.46	2.03	4.08	2.01
Total **	100.0	100.0	....	....	4.188	5.600	1.34	17.06	31.35	1.84
Ear 2										
Aleurone	2.1	5.4	289.0	220.0	1.590	2.090	1.31	6.07	11.88	1.96
Inner endosperm	86.9	76.3	2.8	4.9	.627	.658	1.05	2.43	3.74	1.54
Embryo	11.0	18.3	17.5	18.4	.505	.589	1.17	1.93	3.37	1.75
Total **	100.0	100.0	....	....	2.722	3.337	1.23	10.43	18.99	1.83

* Data for columns so marked taken from (4).
** Data for columns and totals so marked, calculated.

The writers recognize that the use of concentration vs absolute contents is a moot question. The absolute values per kernel-part presented by Teas (4), however, also support the need for a physiologic rather than a morphologic explanation. This is shown in table I, which presents Teas' data (in the columns designated by *), together with some values calculated from them (columns designated **).

Consider, for example, the μg per kernel-part as reported. Although the aleurone contributed most to the total excess of the sugary kernels, all other parts also contributed to that excess. This is more clearly shown by putting the contributions on a ratio basis of *su/Su* as shown in the table. Teas (4, p. 818) states: "Inasmuch as an ear segregating for sugary and normal kernels shows higher niacin content in both whole and degermed sugary kernels than in the normals, the

every kernel part that requires a physiologic explanation and it was discussions of possible reasons for such differences in concentration (1, 3) which Teas (4) concluded were rendered needless by the thicker aleurone he found in sugary kernels. That conclusion seems unwarranted: On the contrary, his data further emphasize the need for a physiologic explanation since they show larger contributions from all individual kernel parts both on an absolute and concentration basis.

In its possible bearing on such an explanation, attention is called to the relation between niacin concentration in the starchy and sugary kernels of individual segregating ears of different crosses as shown by Richey and Dawson (3, tables IX and X). The average excess niacin in the sugary kernels from crosses of Tx 61M, a low-niacin starchy corn, with three dif-

ferent sugary corns was 10 μg per gram. The comparable average for crosses of T13, a high-niacin starchy corn, was 23 μg per gram. Furthermore, the coefficient of correlation for the concentration of niacin in sugary and starchy kernels from individual segregating ears of these crosses was 0.95 with 28 D/F. The *su* gene thus exerted a more or less consistent influence in increasing the niacin proportionally to the level otherwise established. That level presumably was determined by the multiple-factor system demonstrated by Richey and Dawson (2). The larger excess brought about by *su* when the underlying level was higher seems physiologically significant, requiring more than a difference in thickness of aleurone for explanation.

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A DEVICE FOR CONTINUOUS AERATION AND NUTRIENT RENEWAL OF ENTIRE PLANTS UNDER STERILE CONDITIONS[1, 2]

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Several methods for the sterile culture of whole plants have been described in recent years. In some of these (2, 4, 6, 14) only the roots are maintained in a sterile medium. In others (3, 5, 7, 11, 12, 13, 15, 16) the entire plant is enclosed. None of these techniques for aseptic culture of enclosed whole plants provides concurrently for continuous flow of the nutrient solution and for forced aeration, although such a method for excised roots has been reported (9). A simple device which accomplishes both of these purposes is described here. It can be assembled from standard items of glassware and other supplies which are available in most laboratories or from supply houses. The apparatus has the further advantage of being usable either as a single unit or, by T-tube connections, as a battery of units interconnected to air line and nutrient solution reservoir. A set of as many as four assembled units may be sterilized in a reasonably large autoclave without dismantling. While the device is best suited to seedlings, small plants like *Baeria chrysostoma* (13) may be grown throughout their life cycle. The technique has been developed to aid in a study of the effects of pure cultures of microorganisms on the uptake of mineral nutrients, but it is adaptable to many other types of cultures.

One culture-chamber unit (fig 1) includes the following parts: (1) a 500-ml tubulated pressure (filter) flask, (2) a 38 × 300 mm culture tube, (3) a 21-cm length of 6 mm O.D. glass tubing which interconnects the pressure flask and the culture tube, (4) an 80 × 30 mm open-ended glass cylinder containing 3 mm or 5 mm glass beads and resting on the rubber stopper which is used to close the culture tube, and (5) a ¼ inch O.D. T-shaped glass connecting tube, with one arm of the "T" inserted through the 2-hole stopper which closes the culture tube, the other arm joined by rubber tubing to a short length of capillary glass tubing (nutrient solution inlet), and the leg of the "T" connected by rubber tubing to a second length of capillary glass tubing (air inlet). Because of the desirability of sterilizing the unit without disassembling it, all rubber stoppers and tubing should be resistant to steam sterilization and all glassware should be of pyrex or other heat-stable glass.

Several parts of the apparatus serve dual purposes. The heavy glass pressure flask is both a support for the culture device and a sump for the flowing nutrient solution. The glass tubing which elevates the culture tube above the pressure flask also provides an overflow for the nutrient solution in the culture tube and an air outlet. The glass beads act both in promoting aeration of the nutrient solution and in anchorage of the roots. The T-shaped connecting tube permits simultaneous entry of air and nutrient solution into the culture tube, although rates of flow of air and nutrient solution may be independently regulated.

When the device is in operation, nutrient solution enters the T-shaped connecting tube from the lower arm of the "T," air enters from the leg of the "T," and both air and nutrient solution pass together through the other arm of the "T" into the culture tube. It has been found that a more even flow of solution into the culture chamber is obtained by this arrangement than if air enters from below. Sterile air and sterile nutrient solution enter the culture chamber at the base of the open-ended glass cylinder holding glass beads. When the nutrient solution has risen to a height just slightly below the rim of the cylinder holding the beads, it drains from the culture chamber

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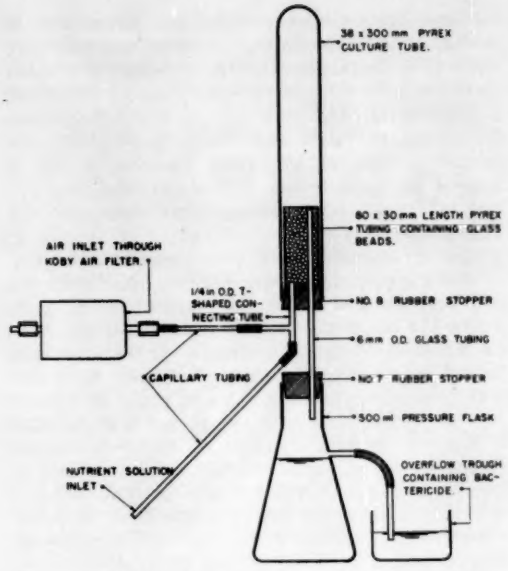
38 x 300 mm PYREX CULTURE TUBE.
80 x 30 mm LENGTH PYREX TUBING CONTAINING GLASS BEADS.
AIR INLET THROUGH KOBY AIR FILTER
1/4 in O.D. T-SHAPED CONNECTING TUBE
NO. 8 RUBBER STOPPER
6 mm O.D. GLASS TUBING
CAPILLARY TUBING
NO. 7 RUBBER STOPPER
500 ml PRESSURE FLASK
NUTRIENT SOLUTION INLET
OVERFLOW TROUGH CONTAINING BACTERICIDE

FIG. 1. Cross-sectional diagram of sterile-culture unit.

through the glass tubing into the flask below. Air, after bubbling up through the nutrient solution along the surfaces of the beads, escapes through the same tube which drains away the nutrient solution. For short growth periods, an air outlet from the pressure flask may be provided by plugging the tubule with sterile cotton or glass wool. For longer periods, the flask may be drained through the tubule as an overflow by connecting it with rubber and glass tubing whose outlet is submerged in a trough containing a bactericide.

In experiments using this sterile culture device, both gravity and pressure-regulated flow of nutrient solution have been employed. With gravity flow, the rate of nutrient renewal decreases with the reduction in hydrostatic head as the reservoir is emptied. If closer control is required, a satisfactorily constant head may be maintained by regulation of air pressure inside the nutrient solution reservoir. This is accomplished as follows. Air from a small Wil-Nes electric aquarium pump enters a pressure equalizing bottle through a Koby air filter (Koby Corporation, Boston) and a one-way valve. One air outlet from the pressure bottle leads to the nutrient solution reservoir and another to a small bottle containing mercury. As pressure in the system increases, mercury in the small bottle is forced up a capillary tube where at the desired maximum pressure it closes a circuit through an electrical contact and relay to shut off the air pump. As pressure in the system decreases, mercury falls in the capillary, breaking the circuit, and the air pump is turned on. Humphries (9) has diagramed a control of similar type. With gravity flow, it may be necessary at the outset to apply slight vacuum to one of the pressure flask air outlets to set the nutrient-solution flow in motion.

With a multiple setup of several culture chambers, some difficulty may be experienced in obtaining a satisfactorily uniform rate of flow of nutrient solution through all culture tubes, because of varying resistances in the system. This can largely be overcome by using capillary tubing of slightly different internal diameters for the nutrient solution inlets. In preliminary flow tests using water, the capillary tubes are interchanged as necessary to equalize flow to the desired degree. During the course of an experiment, if differences in flow rate develop among culture units, they may be corrected by use of open-sided Hoffman clamps. The nutrient solution flow rate may be regulated by placing a clamp in the nutrient solution line of an individual culture unit. It may also be adjusted by using a clamp to increase or decrease the rate of air flow to the unit, since the air flow rate to some extent affects the rate of solution flow.

It is generally easier to maintain uniform flow of nutrient solution among culture tubes at 100 ml or more per culture per day, although considerably lower rates of flow are adequate for plant needs. Rates of air flow have not been measured, but no more air is pumped through the cultures than the minimum necessary to permit adjustment for uniform flow rates among cultures.

With a continuous flow of nutrient solution, it is possible to maintain quite low concentrations. One fifth strength Hoagland (8) solution has been found to give excellent plant growth. A stable form of iron is essential both to avoid the necessity of adding iron to the nutrient solution during the course of an experiment and to reduce the formation of precipitates which tend to clog the capillary nutrient solution inlet. The chelated EDTA iron complex of Jacobson (10) is satisfactory, although use of an equivalent amount of iron as ferrous ammonium sulfate instead of ferrous sulfate in preparation of the complex makes a much more stable stock solution, and the iron seems less precipitable by the pH changes caused by contact with roots. One of the newer sequestrants, diethylenetriamine penta acetic acid (Alrose Chemical Company, Providence, R. I.), is also preferable to the more commonly used ethylenediamine tetra acetic acid (EDTA).

The sterilization of the culture chambers, either singly or in a battery, is accomplished as follows. A Koby air filter is connected to the air inlet of the culture chamber, a tubular fritted glass filter of ultrafine porosity (Corning No. 35000) joined to the nutrient solution inlet, and all air outlets are plugged with cotton or glass wool. The assembled apparatus is then autoclaved at 15 pounds pressure for 30 minutes. The air filter may then be hooked to an air line, and the bacterial filter submerged in a reservoir of nutrient solution. The nutrient solution filter is not necessary if the nutrient solution itself is sterilized and care is then taken in aseptically joining the nutrient solution inlet of the culture chamber to the nutrient solution reservoir. As a double check against penetration of the filter by microorganisms, the nutrient solution is passed through an 8 inch length of quartz-glass tubing

exposed to continuous ultraviolet illumination of principally 2537 Å wave length.

The chief possibility of contamination in this device, as with most others, is through introduction of fungal spores or bacteria into the culture chamber along with the seed or seedling. Fungal contamination is more readily controlled than is bacterial contamination. If absence of growth of microorganisms on selective bacterial or fungal media inoculated with nutrient solution effluent is used as evidence of sterility, all cultures in some later tests were apparently free of fungi, but not more than 70 % of these later cultures were free of bacteria. Their complete freedom from fungal contamination seems to indicate, however, that if bacteria-free seeds or seedlings are introduced into the culture chambers, the apparatus as described here will keep them sterile.

Two methods of starting plants in the culture tubes have been used with the aseptic culture setup described here. One method involves the surface sterilization of seeds with hypochlorite solution under reduced pressure, the thorough rinsing of the seeds with sterile water, and the aseptic transfer of the seeds to the culture chamber, where they are allowed to germinate while resting on the glass beads. This method has two distinct disadvantages, first that all seeds may not germinate, thus eliminating some cultures, and second that microbial contamination is not easily detected. The second method has been found to be more satisfactory. In this method, the seeds are surface-disinfected as before, but then are transferred aseptically to sterile nutrient-agar media in petri dishes where they are germinated. Only the healthy seedlings which show no evidence of contamination are then transferred aseptically to the culture chamber where their growth continues under sterile conditions. Care must be taken to orient the seedlings properly on the glass bead support. Otherwise the developing roots are delayed in reaching firm anchorage.

Checks for contamination during the progress of an experiment may be made either by plating out the effluent from the culture chamber on media which favor rapid appearance of bacterial or fungal colonies, or more conveniently, if specific organisms are not to be identified, by merely placing a small quantity of glucose in the bottom of the pressure flask before the apparatus is autoclaved. Nonsterile cultures quickly develop cloudiness in the overflow nutrient solution if glucose is present.

As with other sterile-culture devices in which the entire plant is enclosed, the apparatus described here poses the problem of growth of plants in an atmosphere of high humidity. An overhead bank of 40-watt fluorescent lamps, spaced at 2 inch intervals, with a ratio of five "soft white" to one "daylight" lamp, has been found to provide adequate illumination (about 1000 fc at the top of culture tubes) without evidence of excessive heating. But even in an air-conditioned room in which temperatures are closely controlled, it has been found that there is some condensation of water on the inner walls of the culture tubes, especially in tubes located at the border of the bank of overhead lights. However, this has not seemed to produce abnormal seedlings, even in cultures which are found to be contaminated. The high humidity might nevertheless be expected in some cases to complicate experiments in which pure cultures of microorganisms are deliberately introduced. Seedlings generally have been maintained in the growth chambers for two to four weeks in apparently normal condition, and culture for longer periods seems limited mainly by lack of growing space.

Certain modifications of this device would be useful under special conditions. For very small seeds and seedlings, the filter paper cone support described by Audus (1) might advantageously be substituted even for the small (3 mm) glass beads. If the plant is to be introduced into the culture chamber as a seed rather than as a seedling, the seed could be inserted into the culture tube with less risk of contamination if dropped through the hole of a rubber stopper closing the top of a glass cylinder of the type used by Loo (13), than if the entire tube must be detached to permit insertion of the seed as is done here. However, the stopper at the top of the tube would reduce the effectiveness of overhead lighting.

The author is indebted to Mr. M. S. Engel for assistance in certain phases of this work, to the Alrose Chemical Company for furnishing several iron-complexing sequestrants used in the study, and to Dr. Philip A. Munz of the Rancho Santa Ana Botanic Garden, Claremont, California, who supplied seeds of *Baeria chrysostoma*.

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FIXATION OF N_2^{15} BY EXCISED NODULES[1, 2]

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Consistent fixation of nitrogen by excised soybean root nodules has been demonstrated recently by Aprison and Burris (2), although previous experiments (4, 7, 8) had yielded positive results only occasionally. The success of the present method is attributed to the short time lapse between picking the nodules and placing them under a gas mixture containing N_2^{15}, and the subsequent analysis of only the acid soluble portion of the nodules for N^{15}. Lincoln variety soybeans were used originally (2), and the present paper describes an extension of the testing method to excised nodules from other varieties of leguminous plants.

The methods used were modified only slightly from those already described (2). The nodules were picked and sized through a cascade of 4 plastic cups with perforated bottoms which would retain nodules of the following sizes: Size 1, over 6 mm in diameter; size 2, between 5 and 6 mm in diameter; size 3, between 4 and 5 mm in diameter; size 4, less than 4 mm in diameter. The screened nodules were washed in ice water, rapidly weighed and transferred to Warburg vessels containing 0.5 ml H_2O, and gassed with a mixture of 10 % N_2^{15}, 20 % O_2, and 70 % He. The N_2^{15} contained 31 atom % excess N^{15}. The flasks were shaken for 1 or 2 hours at temperatures from 22 to 24°C. The nodules then were ground with 5.0 ml of 3 *N* HCl. The mortar was rinsed with two 5 ml portions of 3 *N* HCl, and the mixture was centrifuged. The supernatant was subjected to Kjeldahl digestion; the ammonia was distilled, converted to N_2 with alkaline hypobromite, and analyzed for N^{15} concentration with a Consolidated-Nier mass spectrometer.

Table I records the fixation of N_2^{15} by excised nodules from field grown legumes. Cowpeas and three commercial varieties of soybeans were tested. The best fixation was observed with Lincoln variety soybeans planted early in the season. The same variety planted late in the season under less favorable conditions showed inferior nodule growth and poorer fixation by the excised nodules. Only two results with Blackhawk approach the best fixation observed with Lincoln, and Hawkeye variety was inferior to both the other varieties. Cowpea nodules gave distinctly less fixation than any of the soybeans, although the nodules were well developed and red-centered. Decreased

TABLE I

FIXATION OF NITROGEN BY EXCISED NODULES * FROM FIELD GROWN LEGUMES

VARIETY OF PLANT	DATE OF PLANTING	AGE AT HARVEST, DAYS	SIZE OF NODULES (SEE TEXT)	ATOM % EXCESS N^{15} OF REPLICATES
Glycine Max, var. Lincoln (soybean)	5/8	63	2	0.241; 0.248
	5/8	71	2	0.410; 0.482
	7/26	63	3 **	0.079; 0.086
Glycine Max, var. Blackhawk	5/13	59	2	0.077; ....
	5/13	59	3	0.062; ....
	5/28	64	2	0.142; 0.146
	6/6	53	2	0.151; 0.151
	6/6	59	3	0.137; 0.112
	6/23	44	3	0.111; 0.105
	6/23	56	2 **	0.293; 0.381
Glycine Max, var. Hawkeye	5/13	59	3	0.190; ....
	5/28	64	2	0.098; 0.102
	6/6	53	2	0.123; 0.146
	6/23	56	3 **	0.087; 0.136
	6/23	63	2 **	0.065; 0.067
Vigna sinensis (cowpea)	5/28	44	2	0.016; ....
	5/28	44	3	0.021; 0.056
	5/28	64	2	0.030; 0.091
	5/28	70	3	0.070; 0.065
	5/28	70	3 †	0.031; 0.087
	6/6	53	2	0.037; 0.127
	6/23	44	3	0.045; 0.031

* 1 to 3 gm wet weight of nodules per flask; temp., 22 to 24°C; nodules washed and 0.5 ml H_2O added per flask; time of exposure to N_2^{15}, 2 hours.

** Exposure for one hour.

† These nodules were sliced; no water was added to the slices.

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[3] Predoctorate fellow of the National Science Foundation.

permeability through the thick cortical layer present in cowpea nodules may account for their lower activity, for nodule slices gave nearly as good fixation as whole nodules. In contrast, soybean nodule slices fixed only one third to one fifth as much nitrogen as whole nodules (3).

Table I also illustrates that nodules of nearly the same age, but from different plantings or locations in the field, showed marked variation in their fixation. The size of the nodules was a more important variable than the exact age of the plant in controlling the amount of nitrogen fixed.

Table II shows the results of experiments with nodules from a variety of greenhouse grown legumes. The plants were grown during the spring months from inoculated seed in pots filled with sand. The plants were supplied weekly with a nitrogen-free nutrient solution (6). All exhibited vigorous growth and matured rapidly. Excised Lincoln soybean nodules fixed nitrogen at rates comparable to those observed with field grown plants. Nodules from Canada field peas also showed a high level of fixation, as did those from lupines and alfalfa. Sesbania and Alaska pea nodules also fixed at good rates in some trials. The excised nodules of sweet pea, green bean, and clover fixed lesser but readily detected amounts of N_2^{15}. Earlier (4) it was suggested that, "a single sample should exceed the air control by 0.04 to 0.05 atom % before fixation is indicated." The higher precision and accuracy of our present mass spectrometer conservatively insures positive results with a value of 0.02 atom % N^{15} excess.

TABLE II

FIXATION OF NITROGEN BY EXCISED NODULES * FROM GREENHOUSE GROWN LEGUMES

VARIETY OF PLANT	AGE OF PLANTS, DAYS	SIZE OF NODULES (SEE TEXT)	ATOM % EXCESS N^{15} OF REPLICATES
Glycine Max,	32	..	0.246; 0.356
var. Lincoln	48	2	0.206; 0.119
(soybean)	48	2	0.148; ....
	48	3	0.026; 0.040
Pisum sativum,	32	..	0.095; 0.053
var. Alaska	32	..	0.043; ....
(pea)	48	clump	0.013; ....
	48	..	0.048; 0.053
Pisum sativum,	48	..	0.256; 0.201
var. *arvense*	48	..	0.136; ....
(Canada field pea)			
Lathyrus odoratus,	65	..	0.022; 0.022
var. Spenser			
(sweet pea)			
Phaseolus vulgaris,	32	..	0.014; 0.033
var. Wade	32	..	0.021; ....
(bean)	48	2	0.008; ....
	48	3	0.010; 0.005
Lupinus albus	48	..	0.140; 0.105
(white lupine)	48	..	0.080; ....
	65	2	0.008; 0.019
	65	3	0.007; ....
Trifolium pratense,	55	..	0.049; 0.073
var. Medium red	65	..	0.021; 0.025
(clover)			
Medicago sativa,	55	..	0.118; 0.128
var. Montana	65	..	0.040; 0.052
(alfalfa)	78	..	0.071; 0.046
Sesbania grandiflora,	49	..	0.017; 0.036
Poir.	59	..	0.090; 0.115
	72	2	0.007; 0.027
	72	3	0.004; 0.009

* 0.5 to 2 gm wet weight of nodules per flask; temp., 23 to 24.5°C; time of exposure to N_2^{15}, one hour; nodules washed and 0.5 ml H_2O added per flask.

In all our tests the rate of fixation by excised nodules has increased roughly with increasing size of the nodules from young, actively growing plants. As growth slows with blooming, the rate of fixation also declines. The nodules then soften, their internal bright red color is replaced by brown, and there are accompanying signs of cellular disintegration (1).

In summary, the excised nodules from legumes representing 8 cross inoculation groups (5) have been shown consistently to fix N_2^{15}. Lincoln soybean nodules gave the highest rate of fixation.

We wish to thank Dr. O. N. Allen and Dr. J. C. Burton for the cultures of root nodule bacteria.

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A SIMPLE DEMONSTRATION OF SOME PROPERTIES OF CAPILLARIES[1]

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The apparatus, shown in figure 1, which is similar to that described by Tomaschek (4), allows the direct demonstration of the linearly reciprocal (equilaterally hyperbolic) relation of capillary tension to capillary size. The apparatus consists of two 9" squares of one fourth inch plate glass arranged as a vertical and very narrow "V" trough. The base is a third glass plate. The spacer at the open end of the "V" is a length of 1 mm wire. Heavy rubber bands stretched around the plates provide the necessary pressure. Water poured on the base of the open end of the "V" rises in an equilateral hyperbola if the glass is clean. Light-body lubricating oil has a much slower action but it is more stable and is much better if measurements are to be made of the curve.

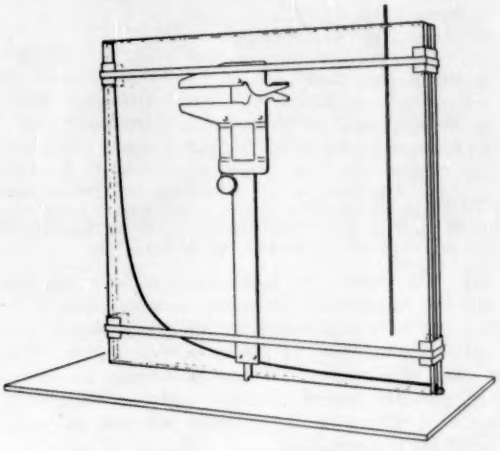

FIG. 1. An apparatus for demonstrating directly that capillary tension is a hyperbolic function of capillary size.

The curve resembles that of soil moisture tension plotted as a function of moisture content (1) and also resembles the curve of diffusion pressure deficit plotted as a function of water content of the soil (2). The lack of exact similarity emphasizes that the taper of capillary spaces in the soil is more complex than the simple straight taper between the glass plates.

The mathematical nature of the curve can be demonstrated easily by making a few measurements on the lower leg of the curve. The upper leg is less satisfactory for measuring because it is very sensitive to imperfections in the system and because of the difficulty of estimating the line of contact between the plates (X = 0). Table I shows a group of measurements made with the depth gauge of an ordinary vernier caliper as shown in figure 1. The height (H) of the curve was measured directly. The width (W) of the capillary was calculated as the product of the distance along the abscissa multiplied by the taper (1 mm/200 mm). Because the product, HW, is constant the curve must be an equilateral hyperbola.

The apparatus suggests a simplification of the usual elementary treatment of capillarity (3, 5). Consider the wedge-shaped body of water between the two plates to consist of a large number of monomolecular sheets of fluid, each suspended vertically from a monomolecular meniscus chain which adheres to the plates. Now, since HW is constant, it is obvious that the load of fluid suspended beneath any meniscus chain in a given system is constant regardless of capillary width. Treatment may now be simplified by one dimension throughout. That is, in the usual treatment adhesive force is considered as acting along the perimeter line, the meniscus is considered a surface, and the suspended fluid is considered a column. In a simplified treatment the adhesive force may be considered as acting on two points, the meniscus may be considered a line, and the suspended fluid may be considered a plane the area of which can be measured directly.

TABLE I

HEIGHT (H) OF FLUID* SUSPENDED BETWEEN GLASS PLATES ARRANGED IN A "V" WITH UNIFORMLY TAPERED SPACING (W)

W	H	HW
mm	*mm*	*mm*^{2}
1.0	6.6	6.60
...	6.7	6.70
0.75	9.0	6.75
...	8.7	6.53
0.50	12.9	6.45
...	13.3	6.65

* Cream-separator oil colored with Sudan III.

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AN ENERGY DEPENDENT INCORPORATION OF AMINO ACIDS INTO THE PROTEIN OF PLANT MITOCHONDRIA [1, 2]

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It has been demonstrated that mitochondrial enzymes from higher plants are able to catalyze the synthesis of the amide bond of glutamine (5), and the peptide bonds of glutamyl-cysteine and glutathione (6) directly from the constituent amino acids. During the course of these studies with isolated mitochondria it was observed that, in addition to performing the syntheses outlined above, the mitochondria were also incorporating the free amino acids into the mitochondrial protein itself. In view of the possible significance of such phenomena for the general problem of protein metabolism in plants, and in view of the complete lack of information on the incorporation of radioactive amino acids into plant proteins, this observation has been examined in more detail. The present communication concerns the apparent coupling of energy production by the mitochondria to the incorporation of amino acids.

Mitochondria were isolated from bean hypocotyl homogenates by the method of Millerd et al (2), with minor modifications as described previously (6). They were incubated with C^{14}-labeled amino acids for one hour at 25°C in potassium phosphate buffer of pH 7.1 plus the additions listed in table I. The reaction was stopped with trichloracetic acid and the mitochondria reisolated by centrifugation at 10,000 × g for 15 minutes. The sedimented protein was washed several times with 5% trichloracetic acid and with 1% potassium hydroxide. The protein was assayed for radioactivity by standard techniques.

Isolated mitochondria incorporated each amino acid incubated with them at slow but measurable rates (table I). The rate of incorporation was markedly increased by the addition of Krebs cycle intermediates and adenylic acid. This increase was strongly inhibited by the respiratory inhibitor, hydrogen cyanide, by anaerobiosis, and by dinitrophenol, which Bonner and Millerd (1) have shown to be a potent inhibitor of oxidative phosphorylation in plants. These results indicate that at least a portion of the incorporation of these amino acids into mitochondrial protein is dependent on the energy produced by respiration.

It has been established that respiratory energy is conserved by the plant in the energy-rich bonds of adenosine triphosphate (1). That adenosine triphosphate can function as the energy donor for amino acid incorporation is indicated by its ability (table I) to replace the Krebs cycle intermediates in increasing incorporation. Incorporation at the expense of added ATP is unaffected by hydrogen cyanide, suggesting that the requirement for oxidative metabolism is, in fact, due to a requirement for an energy supply and that this is bypassed by adenosine triphosphate. The inability of adenylic acid alone to increase incorporation provides further evidence that the energy-rich bonds of adenosine triphosphate are the necessary factor for incorporation.

TABLE I

THE EFFECT OF VARIOUS ACTIVATORS AND INHIBITORS ON INCORPORATION OF AMINO ACIDS BY MITOCHONDRIA

ADDITIONS TO SYSTEM *	μM AMINO ACID * INCORPORATED/GM PROTEIN		
	glycine	*glutamic acid*	*aspartic acid*
1. None	0.03	0.08	0.09
2. Adenylic acid + Mg^{++}	0.04	0.10	0.10
3. Same as no. 2 + citrate	0.11	0.23	0.21
4. Same as no. 2 + α-keto-glutarate	0.10	0.21	0.20
5. Same as no. 2 + malate	0.09	0.20	0.20
6. Same as no. 3 + HCN	0.05	0.11	0.11
7. Same as no. 3, incubated under nitrogen	0.07	0.15	0.14
8. Same as no. 3 + dinitro-phenol	0.04	0.08	0.10
9. Same as no. 2 + ATP	0.20	0.39	0.33
10. Same as no. 9 + HCN	0.21	0.38	0.35

* Concentrations of all additions to system was 0.001 M. Concentration of the added amino acids was 0.01 M. The general conditions utilized for incubation were as described by Bonner and Millerd (1).

It appears, therefore, that the adenosine triphosphate produced during plant respiration may actively participate in the turnover of amino acids in mitochondrial protein. The fact that this turnover occurs at the same time that the mitochondria are catalyzing various respiratory and synthetic functions [oxidation of organic acids (2), phosphorylation of adenylate (1) and synthesis of hexose phosphate (4), acetyl-CoA and acetoacetate (3), and simple amides and peptides (5, 6)] gives a further indication of the complex spectrum of metabolic reactions that are associated with mitochondria.

The author wishes to thank Dr. James Bonner for his helpful comments.

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[2] Supported in part by the Polychemicals Department, E. I. du Pont de Nemours and Co.

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INSERTION TO DISCUSSION, PAGE 186, OF THE ARTICLE BY HULL, WENT, AND YAMADA

It is of interest that Siegel and Weintraub (9) have found that the inhibitory effect of hydrogen peroxide on the activity of IAA in the Avena test could be almost completely reversed by subsequent incubation of the IAA-peroxide mixture with catalase, prior to its application to the coleoptile. (Catalase alone has no effect on IAA response, nor causes curvature in its absence.) Consequently, it must be assumed that the loss of IAA activity in the presence of hydrogen peroxide is not due to auxin destruction, but to some type of interference of the growth response itself. These investigators also noted that organic peroxides from ether acted in the same manner. The removal of these substances however, had to be accomplished by dilution rather than by catalase. Thus we find here a parallel case in which peroxides negate the activity of an auxin without destroying it.

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